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TITLE:

"Treatment and Prevention of Breast Cancer Using Multifunctional Inhibitors of Cholesterol Biosynthesis"

PRINCIPAL INVESTIGATOR:

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RECIPIENT:

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Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)
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Columbia, Missouri 65211-1230

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14. ABSTRACT
Most human breast cancers are hormone responsive, depending on estrogens and progestins for tumor cell proliferation. Initially, hormone-responsive tumors respond to endocrine therapy, however, most human breast tumors develop resistance to currently used endocrine therapeutic protocols. It is therefore essential that we identify additional molecular targets in the signaling pathways that lead to tumor growth if we are to effectively treat and prevent cancers of the breast. Our goal was to identify alternative targets in the pathway leading to the production of cholesterol, which might be regulated with less toxic inhibitors to control the progression of breast disease. Inhibitors of oxidosqualene cyclase (OSC), an enzyme down-stream of HMG CoA-reductase in the cholesterol biosynthetic pathway, effectively arrested breast cancer cell proliferation. In the 2013 Annual Report we discussed several interesting observations establishing that an OSC inhibitor, RO 48-8071 (RO) is an effective anti-cancer compound. These results are now published and appended as a research article in Breast Cancer Research and Treatment, a highly reputable journal. During our second year of research we have continued these studies and shown that combination therapy involving RO and an estrogen receptor-beta agonist is an extremely effective means of treating breast cancer. We found that estrogen receptor beta is induced and, importantly, that the pro-proliferative estrogen receptor-alpha is destroyed by RO. These studies show for the first time that inhibition of cholesterol biosynthesis using OSC inhibitors is a novel and potent means by which to destroy human breast cancer cells, and, furthermore, that a combination of RO with agonists of estrogen receptor beta is a viable treatment option that should be considered for patients who exhibit estrogen receptor in their biopsies. Finally, in the past year we have also established that RO controls not only the activity of estrogen receptor, but also androgen receptor activity. These results are attached and are of particular importance considering that androgen receptor is regarded as a target for triple negative breast cancers for which there are few options available to control the progression of this deadly disease.

15. SUBJECT TERMS- none provided

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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Table of Contents

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The purpose of this research is to investigate whether an inhibitor of cholesterol synthesis (RO) is also an effective therapeutic drug which can be used to control the progression of breast cancer. The effects of RO on a number of different breast cancer cell lines have been examined, as well as its in vivo effect against breast cancer cells grown in xenograft models. Protocols in which the inhibitor is given alone or in combination with other compounds have been employed. Initial results were reported last year. These have now been published and the manuscript is appended. During the past 12 months intensive studies to determine the effectiveness of combination therapy using RO and estrogen receptor beta agonists have been performed. The results are reported herein. In addition, efforts have been made to determine whether RO influences the transcriptional activities of estrogen receptor.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Breast Cancer, Cholesterol inhibitors, therapeutics, Cell viability, apoptosis, estrogen receptor beta, combination therapy, transcriptional activity, androgen receptor.

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

Progress related to Task 1. ***Characterize the impact of RO on estrogen signaling in breast cancer cells. (estrogen receptor alpha=ERα; estrogen receptor beta=ERβ)***

Goals:

a. Determine the effect of RO therapy on cell viability using a number of different breast cancer cells, and normal mammary cells.

b. Determine the level of ERα and ERβ expression in treated cells by Western blot analysis.

c. Determine the effect of RO treatment on estrogen-dependent proliferation of breast cancer cells.

d. Determine whether RO treated ERα positive cells lose their capacity to regulate ERα-dependent gene regulation but retain the ability to regulate ERβ specific genes with ERβ-interacting ligands.
e. Determine whether RO influences transcription of ERα and ERβ genes in breast cancer cells.
f. Determine whether RO influences stability of ERα and ERβ protein in breast cancer cells.

This aim is mostly complete and a manuscript describing the results has been published (appendix). A recently presented abstract at the Annual Endocrine Society Meeting is also appended. In the last year we addressed point e. above and showed that RO influences the transcriptional effects of estrogen receptor, and surprisingly, of androgen receptor as well. These results are reported in attached Figs 1-6. A manuscript describing this data is currently in preparation.

Progress related to Task 2. ***Characterize the in vitro effects of RO mono- or combination therapy on proliferation and apoptosis of breast cancer cells in vitro.***

Goals:
a. Measure apoptosis in breast cancer cells treated with RO alone or RO in combination with ERβ interacting ligands.
b. Determine protein levels of apoptosis related genes (p21, caspase-3, Bcl-2, Bad, Bax) following treatment of cells with RO.
c. Initiate combination therapy, keeping the concentration of one ligand constant while varying that of the other to determine whether there are additive or synergistic effects on apoptosis.
d. Determine mRNA and protein levels of proteins related to apoptosis and angiogenesis, such as p21, caspase-3, Bcl-2, Bad, Bax, and VEGF following treatment of cells with RO in combination with aforementioned compounds.
e. Transfect cells with siRNA to down-regulate ERβ and determine cell viability and response to RO using cell-proliferation assays.
f. Following ERβ and OSC siRNA transfections, test breast cancer cells for lack of sensitivity to RO in order to define a molecular target for mediating RO effects. These experiments will utilize cell viability assays.

A number of studies described in task 2 have been completed and also reported in the attached manuscript. However in the last year we have spent considerable effort to address point c. above and study the effect of combination therapy. Please see attached Figures 7-10 for results obtained. As indicated in task 3, we are making progress with our studies to determine the in vivo effects of combination therapy for controlling breast cancer progression and we have obtained a no-cost extension from DOD officials to continue since more time is needed if we are to elucidate the mechanism behind our observations (also see task 3).

Progress related to Task 3. ***Characterize the effects of RO mono- or combination therapy on progression and prevention of breast cancer cells in vivo in rodent models.***

Goals:

a. Breast cancer cells in matrigel will be inoculated into nude mice (6-8 week-old, female, nu/nu, sc).
b. Tumors will be allowed to reach 100-200mm^3 in size, after which animals will be randomly assigned to groups for treatment with RO or vehicle alone. RO treatment (5-25 mg/kg, iv) will be once a day for 10 days.
c. Experiment in b) will be repeated in vivo using a combination of RO and an ERβ specific ligand, as well as RO and a natural compound with an affinity for ERβ, to determine additive or synergistic effects in reducing in vivo tumor progression.
d. Tumor samples will be collected after the first three injections and then again at the end of the experiment in b. and c. for further analysis by immunohistochemistry. Samples will also be saved in liquid nitrogen for Western blot analysis and RNA isolation.
e. Western Blot and RT-PCR will be used to analyze protein levels and RNA expression of ER, PR , p21, Caspase-3 and VEGF.
f. Immunohistochemistry will be used to quantitate blood vessel density and various antigens indicated in e.

In vivo studies using RO are underway. We have already shown in our previous report that RO is effective against breast cancer (manuscript attached). We now have robust data from experiments conducted in the last year showing that combination therapy with RO and an estrogen receptor beta ligand is an efficient way to treat both hormone-dependent and hormone-independent breast cancers without toxicity (Fig 7A-B). We conducted in vitro combination therapy and utilized liquiritigenin, an ERβ interacting ligand which was most effective in vitro, in our subsequent in vivo studies. These experiments are reported in Figs 7-10. We have taken a one-year no-cost extension to complete immunohistochemical analysis of tumor tissue and determine the mechanism for such a powerful anti-tumor effect. A few other studies in tasks 1 and 2 will also be finalized during the no-cost extension.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

    - OSC expression is not associated with stage of breast disease
    - RO blocks transcriptional activities of both estrogen and androgen receptors
    - RO blocks estrogen receptor alpha more strongly than estrogen receptor-beta
    - RO competes with estrogen for binding to the estrogen receptor though at much higher concentrations, suggesting an allosteric modification of estrogen receptor
    - RO blocks the production of an estrogen regulated gene (progesterone receptor) in breast cancer cells
    - RO does not regulate estrogen receptor at the transcriptional level
    - RO in combination with estrogen receptor beta interacting agonists is a powerful combination which stops the progression of breast tumors both in vitro and in vivo

5. **CONCLUSION:** Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

Our research, with the support of this grant, shows that inhibitors of cholesterol synthesis that target OSC induce tumor cell apoptosis and can therefore be used to prevent the progression of breast cancer cells. In addition OSC inhibitors have off-target effects; they degrade estrogen receptor alpha, a major pro-proliferative protein in hormone-responsive cells, and induce estrogen receptor beta protein, a major factor which reduces cell proliferation. The results pertaining to combination therapy involving RO and estrogen receptor beta interacting ligands are particularly interesting and important since they potentially support the use of lower levels of toxic chemotherapeutic drugs together with RO to bring about tumor regression. Once the studies proposed in the grant are complete, they will yield information vital to determining the suitability of these drugs for use in humans. We propose that it is important to move forward with human clinical trials which we believe could set the stage for the therapeutic use of OSC inhibitors against breast cancer, and potentially save millions of lives worldwide.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press:

http://munews.missouri.edu/news-releases/2014/0617-potential-cholesterol-lowering-drug-has-breast-cancer-fighting-capabilities-mu-researcher-finds/

(2) Peer-Reviewed Scientific Journals:

Liang Y, Besch-Williford C, Aebi JD, Mafuvadze B, Cook MT, Zou X, Hyder SM. (2014) Cholesterol biosynthesis inhibitors as potent novel anti-cancer agents: suppression of hormone-dependent breast cancer by the oxidosqualene cyclase inhibitor RO 48-8071. Breast Cancer Res Treat. 146:51-62.

(3) Invited Articles:

Hyder, S. M., Mafuvadze, B and Besch-Williford, C. (2013) Novel Anti-Angiogenic Therapies using Naturally-Occuring and Synthetic Drugs to Combat Progestin-Dependent Breast Cancer to be published in Cell and Molecular Biology of Breast Cancer, Humana Press, In Press

(4) Abstracts:

Liang, Y., Zou, X., Besch-Williford, C., Johnnes, A. and Hyder , S. M. (2013) Synthetic inhibitors of the cholesterol biosynthetic enzyme oxidosqualene cyclase block proliferation and survival of breast cancer cells. 103rd Annual American Association of Cancer Research Meeting, Washington DC, USA, Abstract #871

Mafuvadze, B., Liang, Y., Hyder, S. M. (2014) Oxidosqualene Cyclase Inhibitor Suppresses Transcriptional Activity of Estrogen Receptor-α in Human Breast Cancer Cells. 16th International Congress of Endocrinology and the Endocrine Society's 96th Annual Meeting and Expo, Chicago, IL. Abstract SAT-0279

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

*Liang, Y., Zou, X., Besch-Williford, C., Johannes, A. and Hyder , S. M. (2013) Synthetic inhibitors of the cholesterol biosynthetic enzyme oxidosqualene cyclase block proliferation and survival of breast cancer cells. 103rd Annual American Association of Cancer Research Meeting, Washington DC, USA, Abstract #871

Mafuvadze, B., Liang, Y., Hyder, S. M. (2014) Oxidosqualene Cyclase Inhibitor Suppresses Transcriptional Activity of Estrogen Receptor-α in Human Breast Cancer Cells. 16th International Congress of Endocrinology and the Endocrine Society's 96th Annual Meeting and Expo, Chicago, IL. Abstract SAT-0279

7. **INVENTIONS, PATENTS AND LICENSES:** List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

Nothing to report

8. **REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates

to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

All the results described in Section 3 are reportable. Some of these have been published (attached manuscript), and additional data will soon be published. The results show a substantial advance towards a potentially new therapeutic protocol for breast cancer which could involve the use of specific cholesterol lowering drugs that target oxidosqualene cyclase. These drugs may be administered with or without additional drugs that target estrogen signaling mechanisms. Evidence for such a possibility comes from our observation that estrogen receptor beta specific ligands appear to enhance the effects of cholesterol lowering drugs. It is possible that such an approach could also prove useful for preventing breast cancer in the first place.

9. **OTHER ACHIEVEMENTS:** This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

   Nothing to report

For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

10. **REFERENCES:** List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

    n/a

11. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

    Please see attached

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on this eReceipt System https://cdmrp.org/Program_Announcements_and_Forms/ and under "Forms" on https://www.usamraa.army.mil) should be updated and submitted with attachments.

MARKING OF PROPRIETARY INFORMATION: Data that was developed partially or exclusively at private expense shall be marked as "Proprietary Data" and Distribution Statement B included on the cover page of the report. Federal government approval is required before including Distribution Statement B. The recipient/PI shall coordinate with the GOR to obtain approval. REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE. It is the responsibility of the Principal Investigator to advise the GOR when restricted limitation assigned to a document can be downgraded to "Approved for Public Release." DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS. See term entitled "Intangible Property – Data and Software Requirements" and https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting for additional information.

Figure 1: Relative OSC mRNA expression in breast cancer at different stages of growth determined using real-time PCR assay
(No significant correlation was obtained)

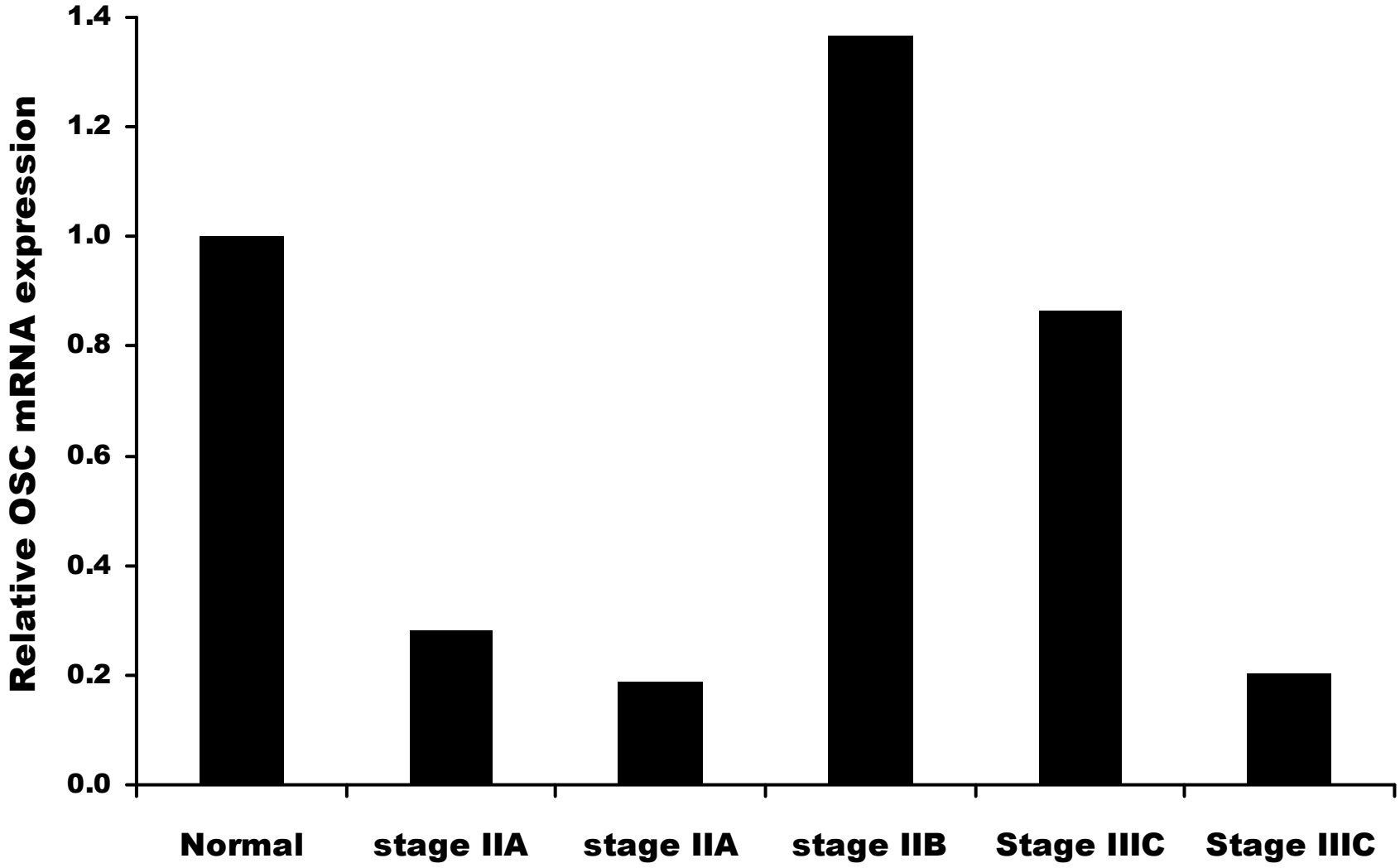


Figure 2: Relative OSC mRNA expression in distinct breast cancer tissues at different stages of growth (Stage I-III).
Results showed no difference in expression between ER/PR positive, ER/PR negative and triple-negative tissues (ER/PR and Her2-neu negative). *triple-negative, **ER/PR negative Her2-neu high; remaining tumors are ER/PR positive

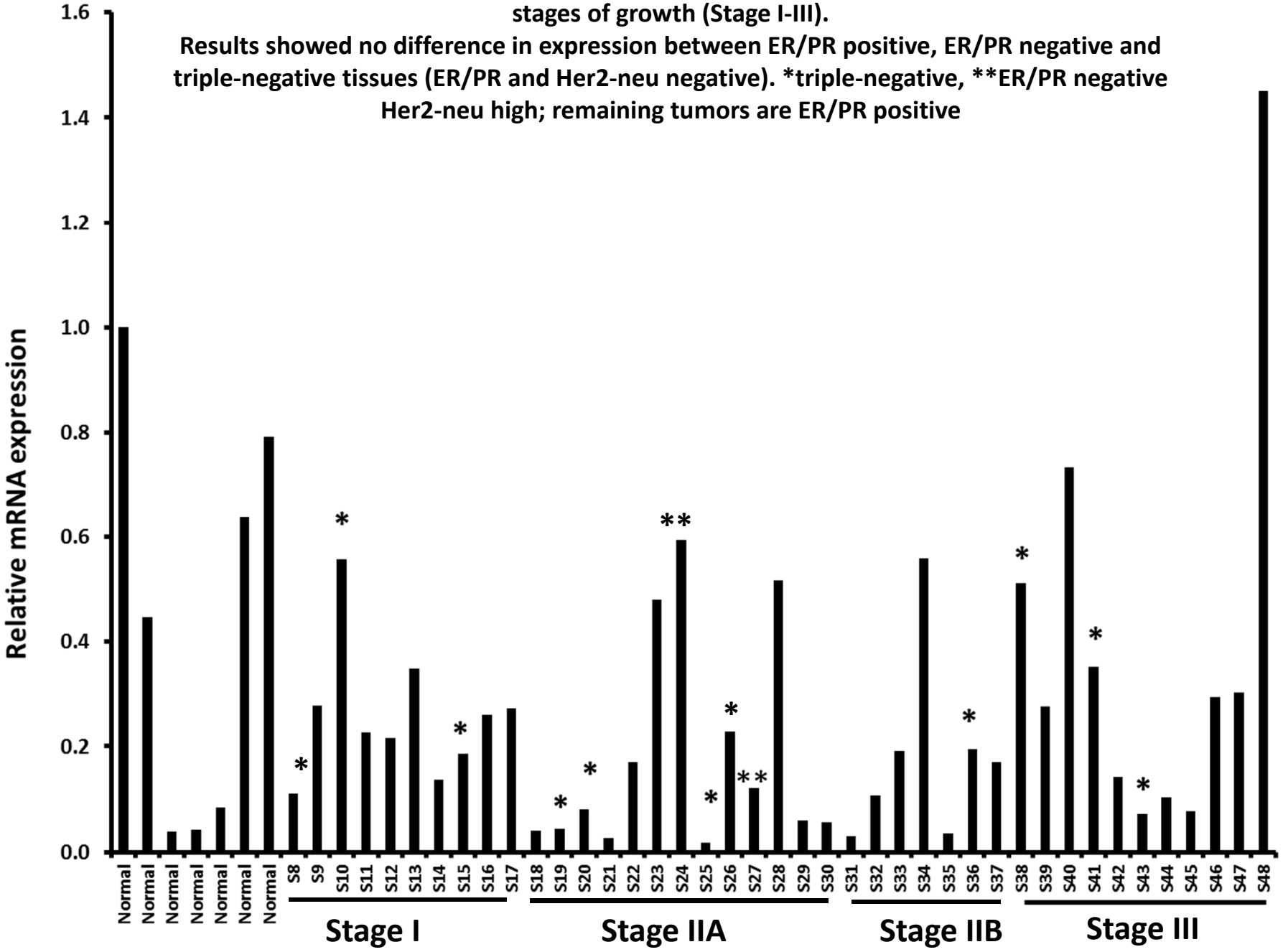


Figure 3A-D: RO significantly inhibits estradiol induced estrogen receptor-mediated transcriptional (luciferase) activity.

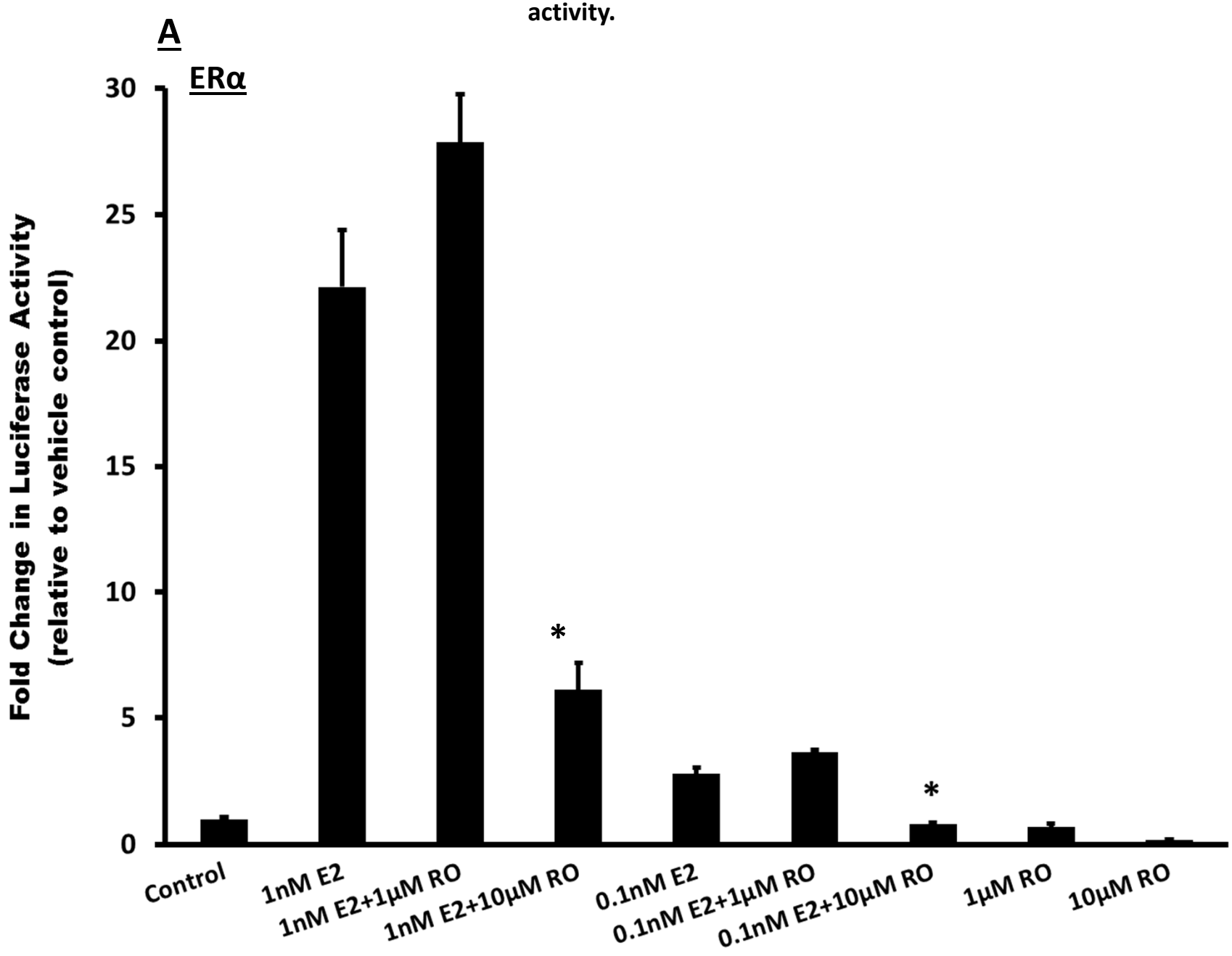


Figure 3B.

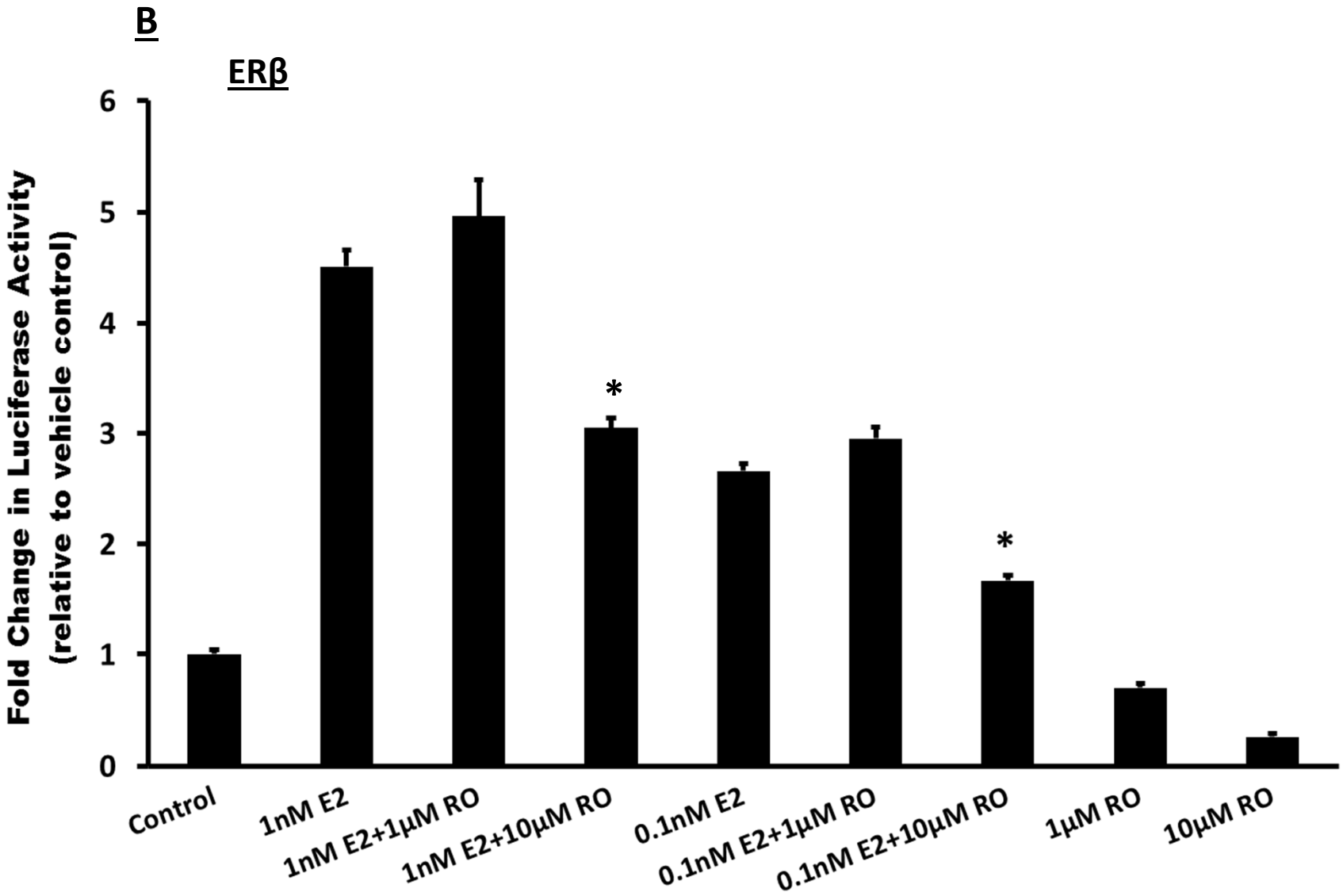

B
ERβ
Fold Change in Luciferase Activity
(relative to vehicle control)
0
1
2
3
4
5
6
*
*
Control
1nM E2
1nM E2+1μM RO
1nM E2+10μM RO
0.1nM E2
0.1nM E2+1μM RO
0.1nM E2+10μM RO
1μM RO
10μM RO

Figure 3C. ICI=ICI 182,780, an anti-estrogen (antagonist)

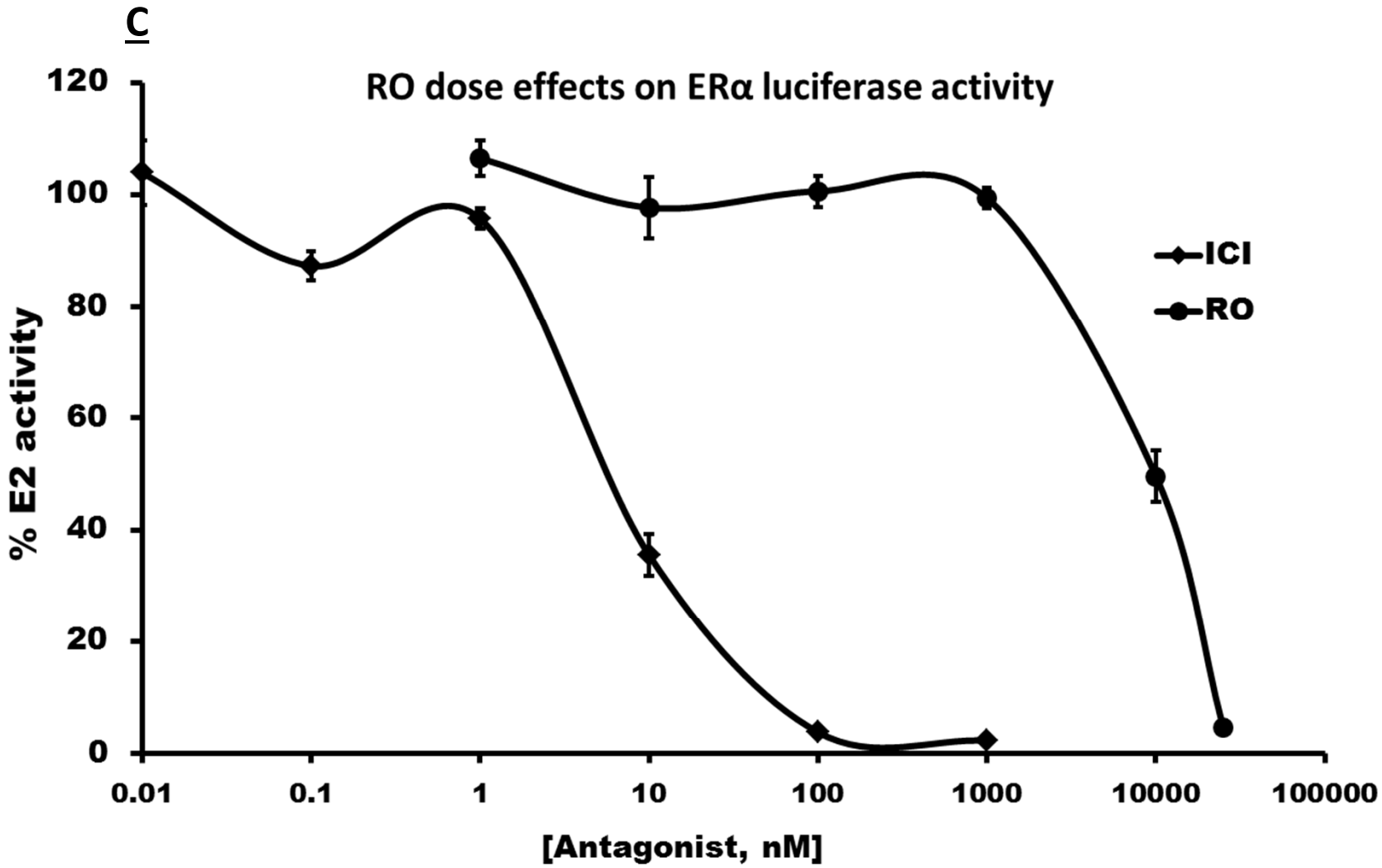
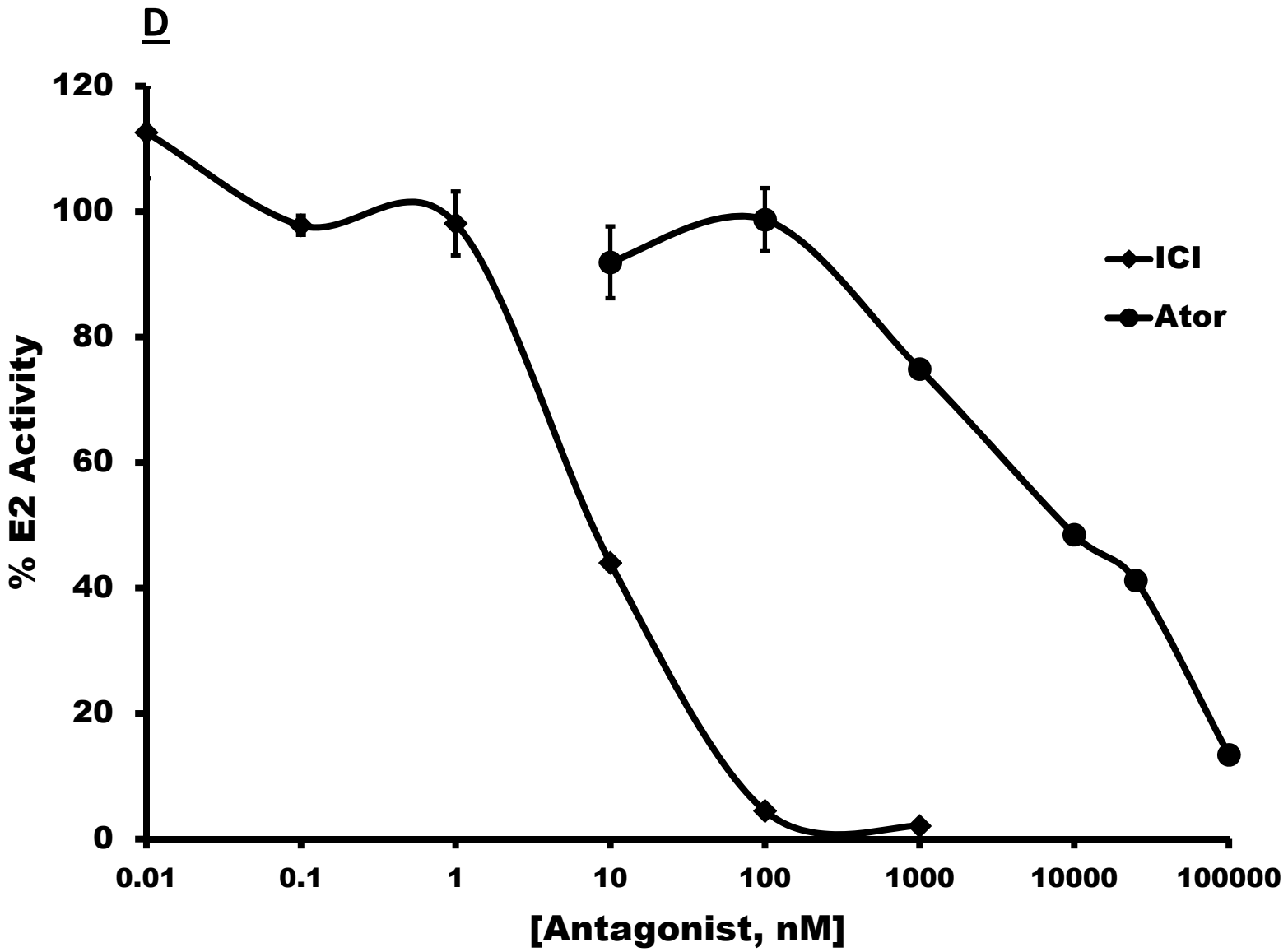


Figure 3D. Ator=Atorvastatin



<u>Figure 4:</u> RO significantly inhibits androgen-induced androgen receptor activity (luciferase). OH-Flut, hydroxyflutamide, an antagonist for androgen receptor; ICI= ICI 182,780, an antagonist for estrogen receptor; Ator, Atorvastatin, a statin used for lowering cholesterol

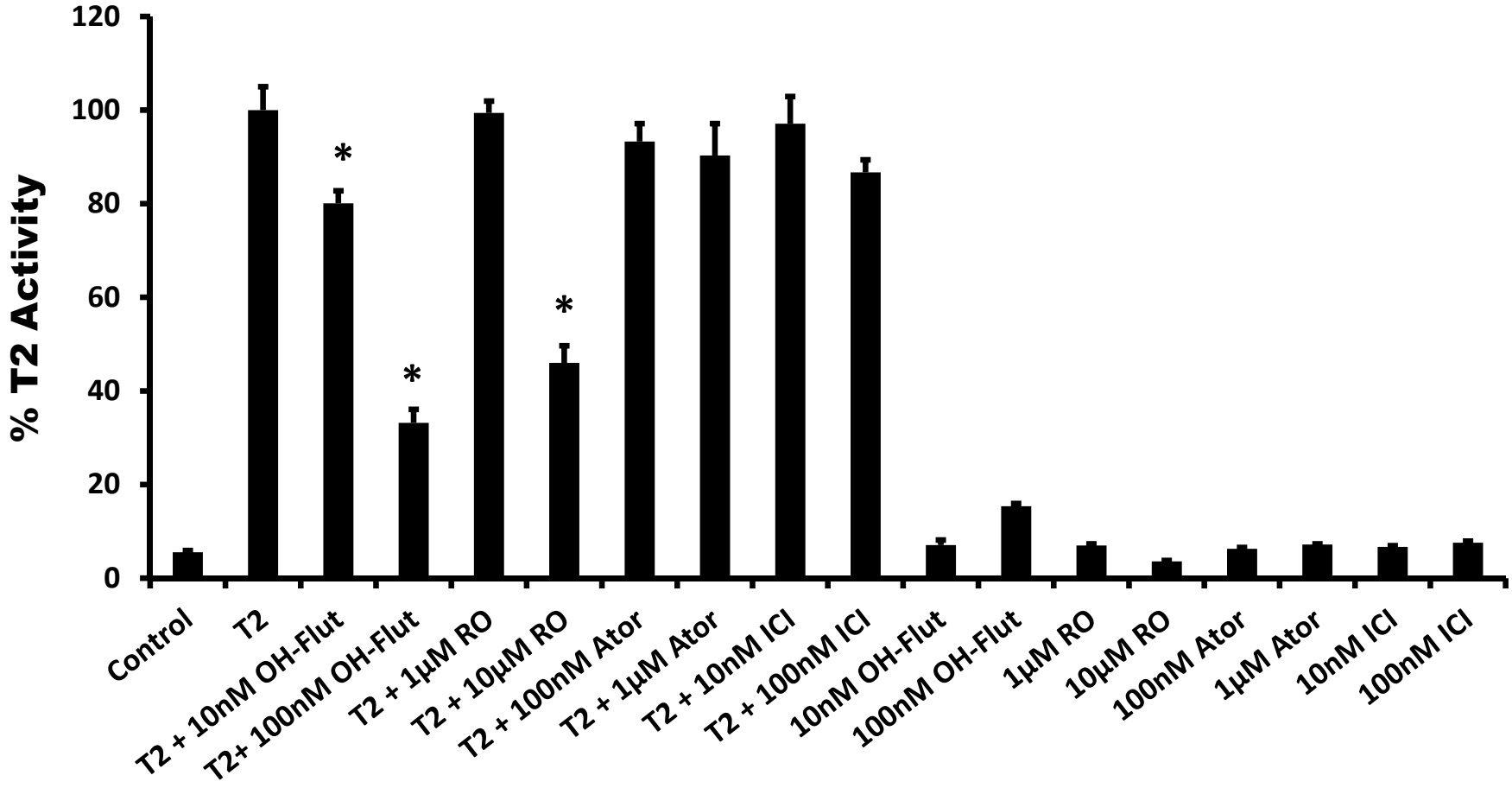


Figure 5. **RO and other compounds tested do not show toxicity effects at the concentrations used. Sta= Staurosporine, an agent that induces apoptosis and kills cells.**

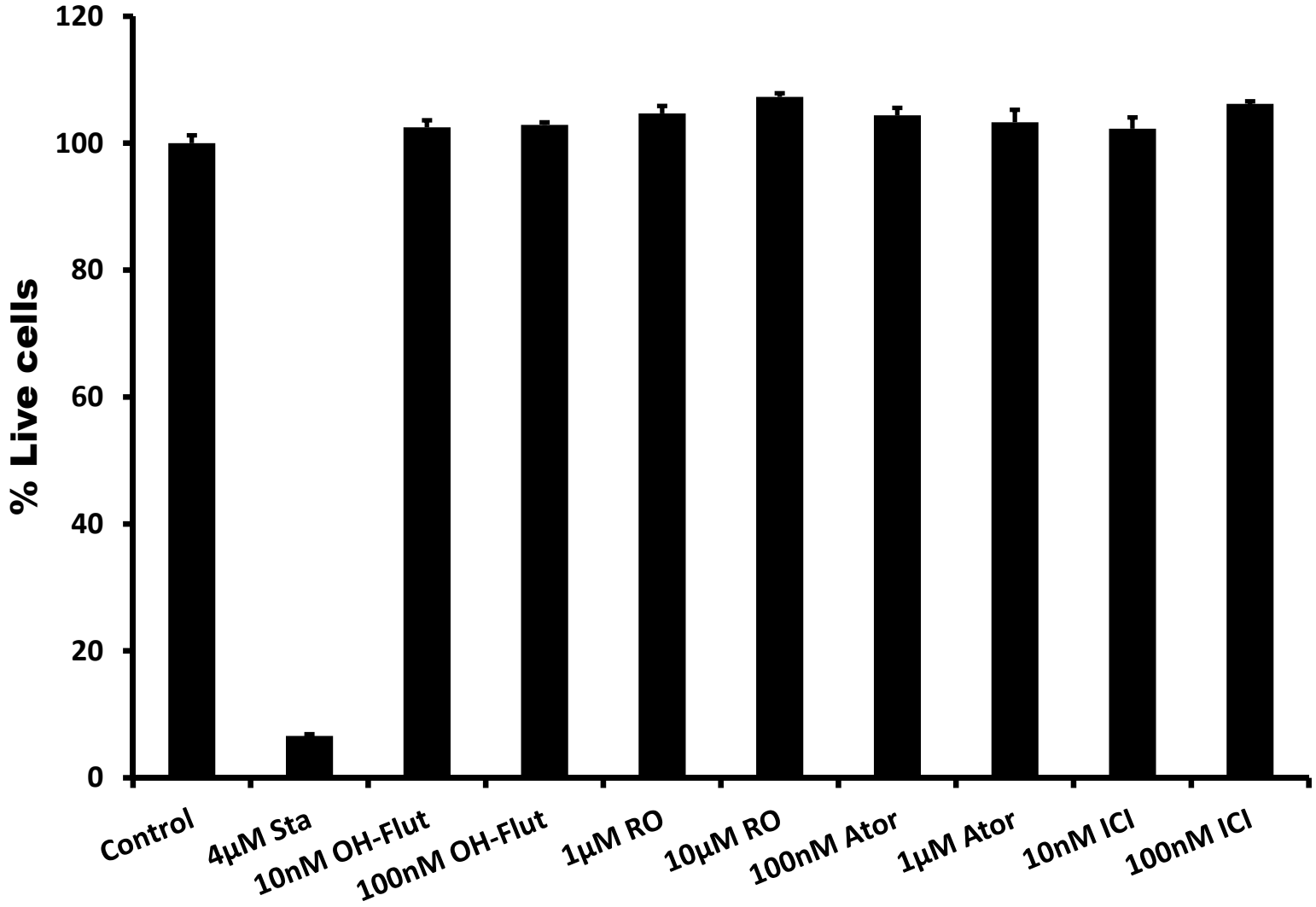


Figure 6A: RO inhibits estradiol –induced progesterone receptor expression in breast cancer cells. E2=Estradiol

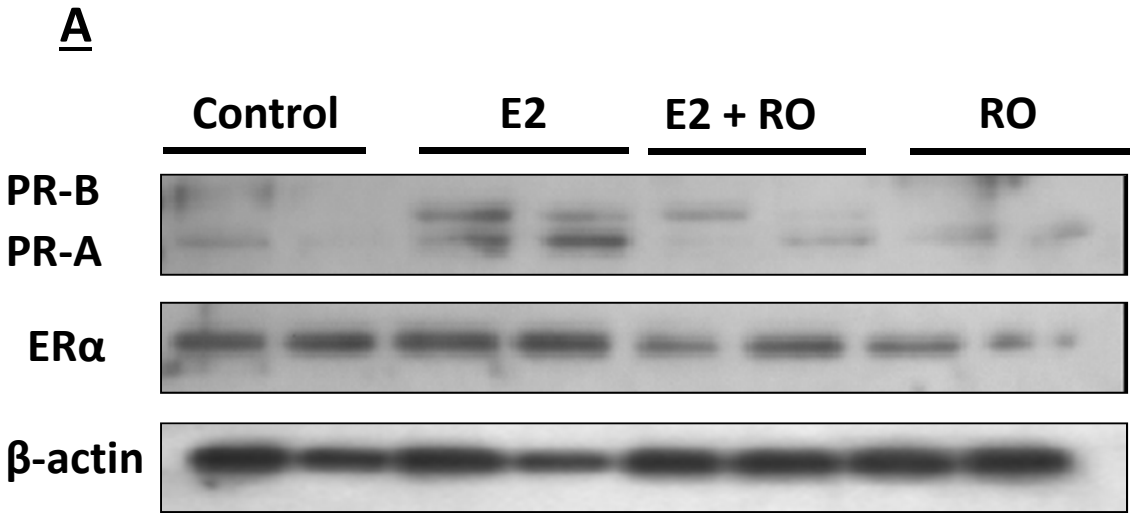


Figure 6B: Relative estrogen receptor mRNA expression in human breast cancer cells

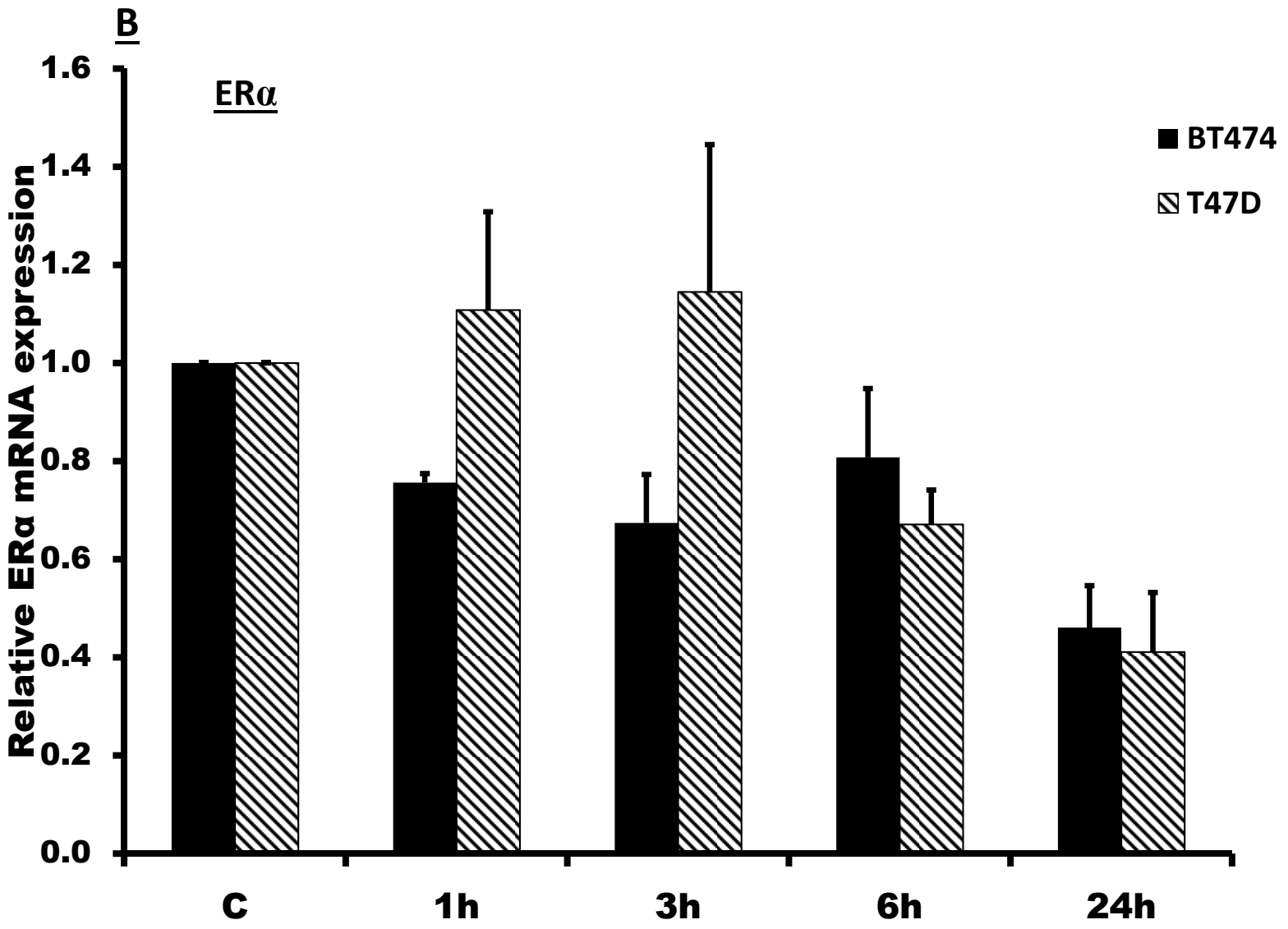


Fig 7A: Effect of 4-OH-Tamoxifen monotherapy on viability of different breast cancer cell lines. Note that sensitivity of MDA-MB-231 were less sensitive to tamoxifen.

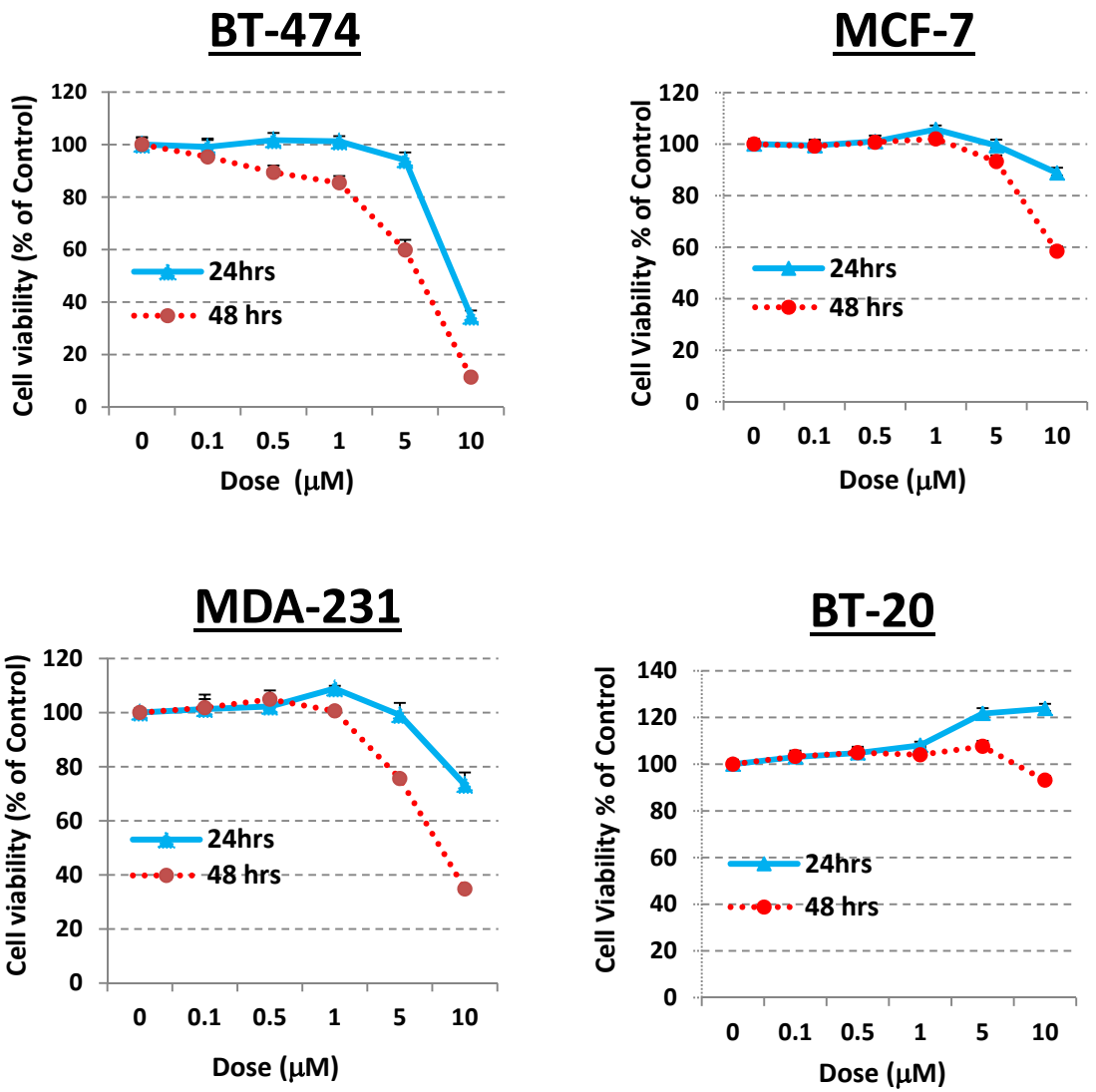
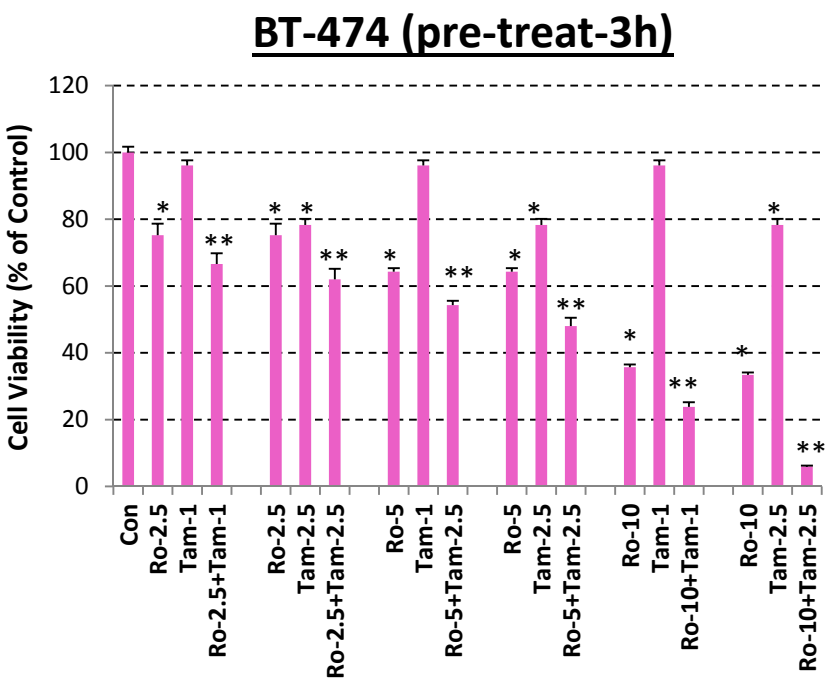


Fig 7B: Effect of combination therapy using Ro 48-8071 plus Tamoxifen on viability of BT-474 and MDA-MB-231 cells. Cells were pre-treated with RO to induce estrogen receptor beta. Comparison with Fig 7A shows that combination therapy is extremely effective against these cell lines. Concentrations are in μM. *, significantly different from control group, ** significantly different from other groups (ANOVA).



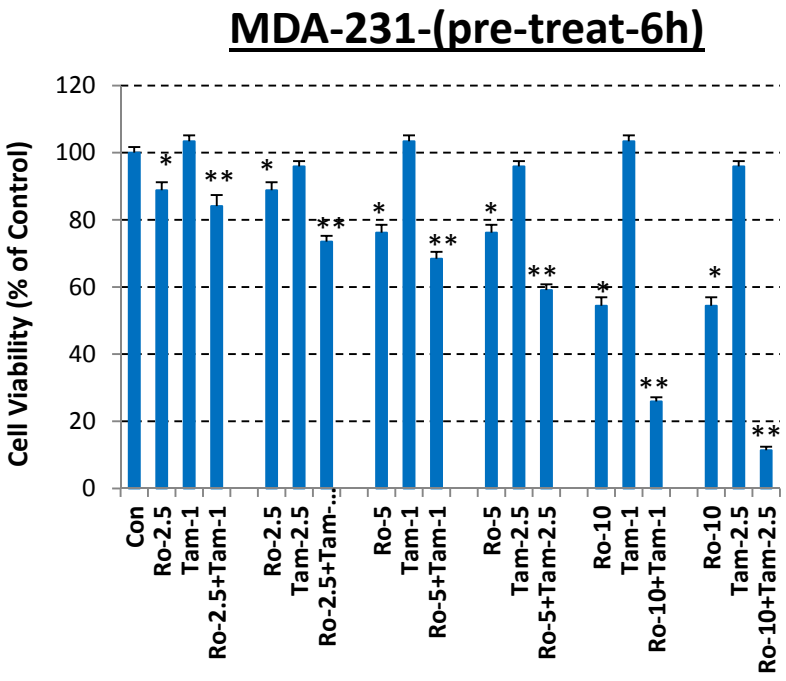


Fig 8A: Effect of Liquiritigenin, an estrogen receptor beta agonist, on viability of different breast cancer cell lines.

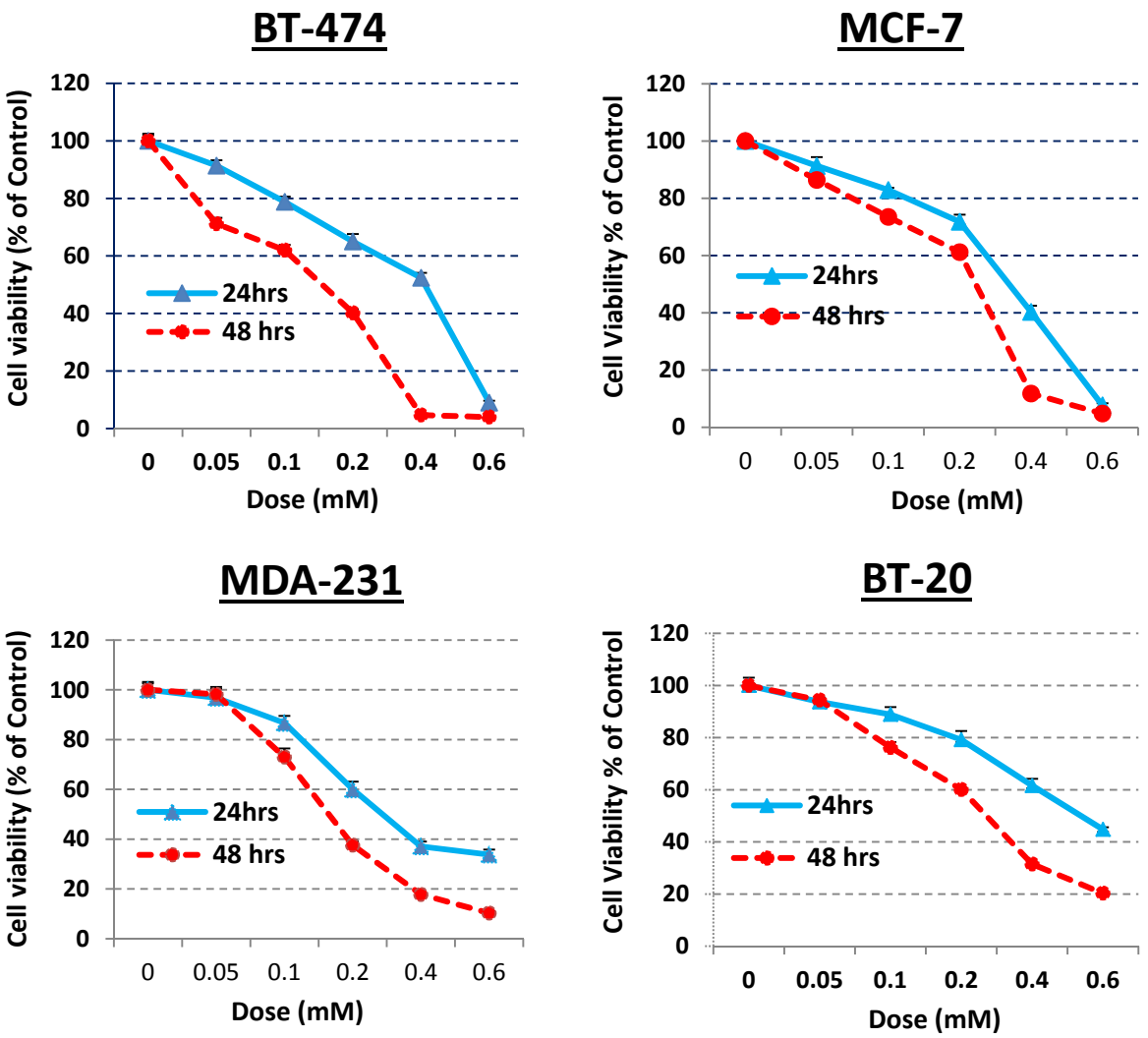


Fig 8B: Effect of Ro 48-8071 plus Liquiritigenin on growth of estrogen receptor alpha positive breast cancer cell lines (treatment 24 h). Cells were pre-treated with RO for 3-6 h to induce estrogen receptor beta and then RO was added. Figure shows that combination therapy is extremely effective against these cell lines. Concentrations are in μM. *, significantly different from control group, ** significantly different from other groups (ANOVA).

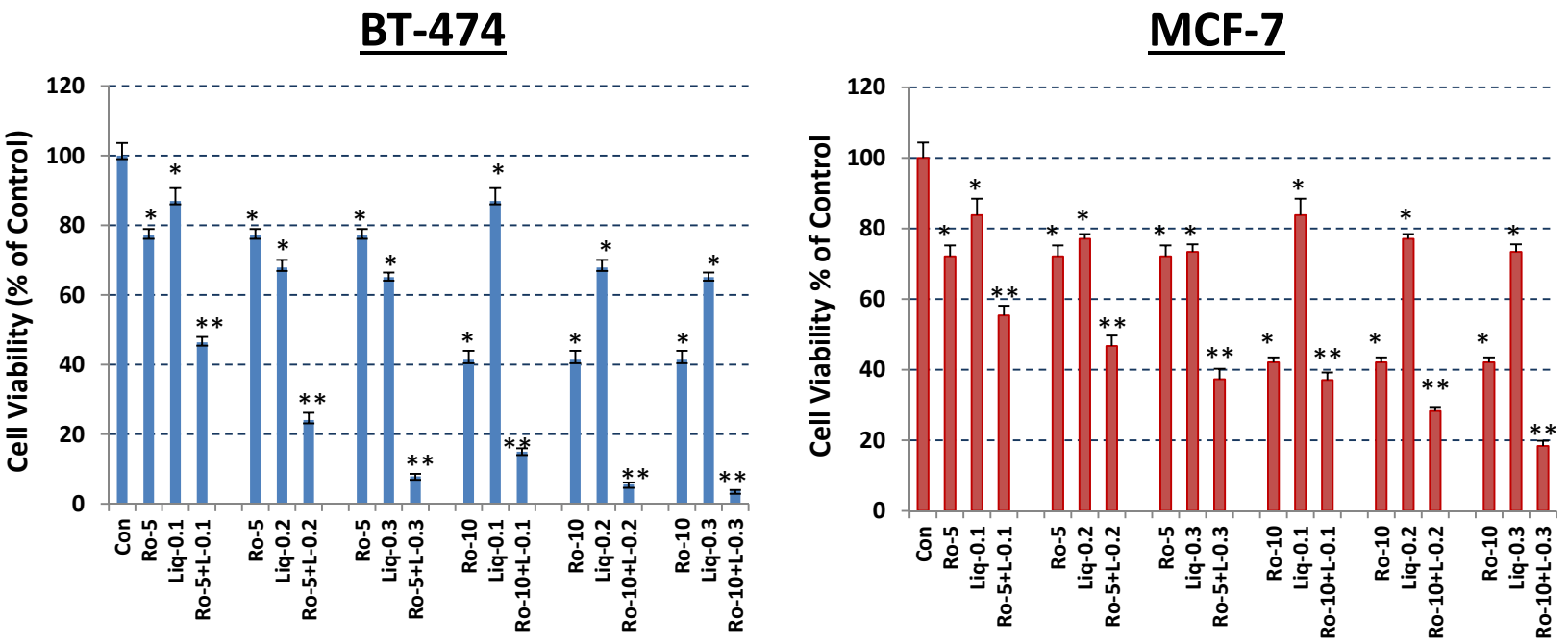


Fig 8C: Effect of Ro 48-8071 plus Liquiritigenin on growth of estrogen receptor alpha negative breast cancer cell lines. Cells were pre-treated with RO to induce estrogen receptor beta and then treatment was continued for another 18 h. Concentrations are in μM. Combination therapy was extremly effective as shown in the figure below. *, significantly different from control group, ** significantly different from other groups (ANOVA).

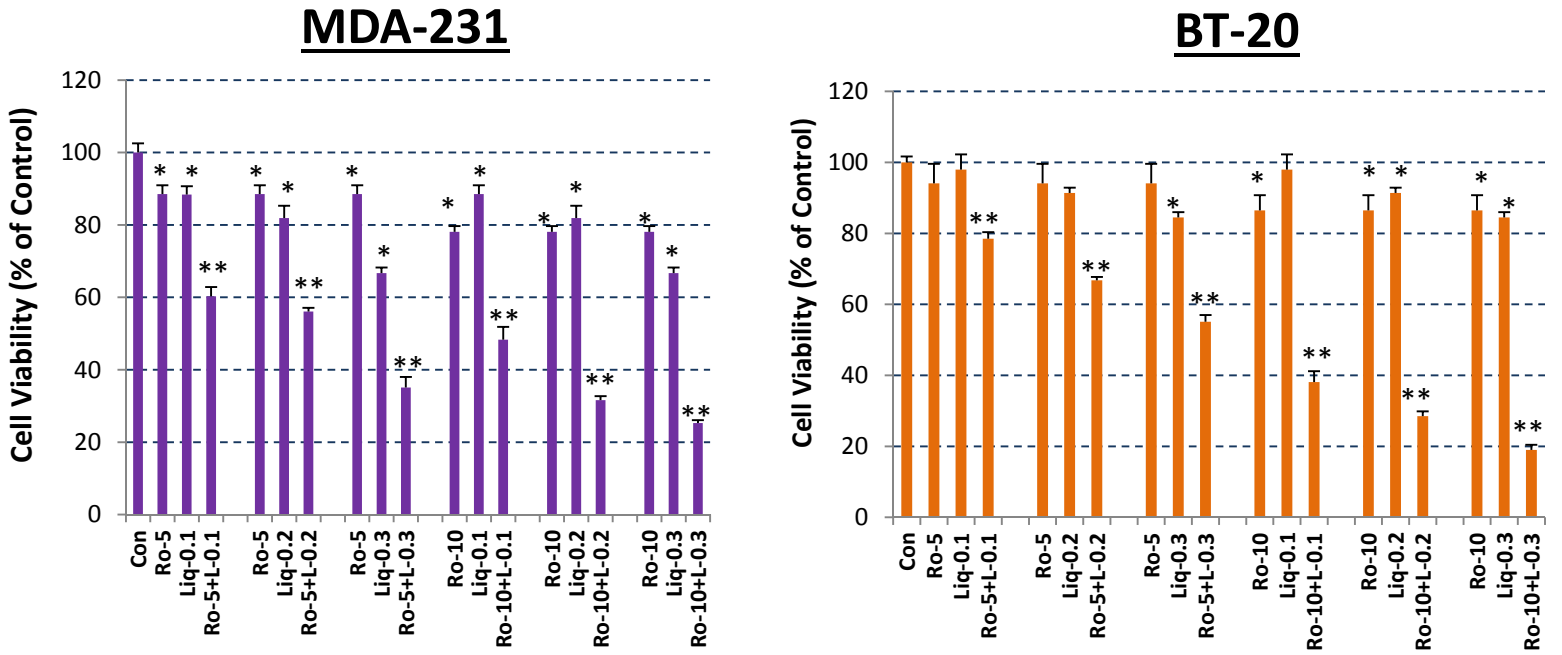


Fig 9A: Effect of Ro 48-8071 combined with Liquiritigenin on in vivo growth of BT-474 xenografts. Treatment was with RO 10 mg/kg iv + Liquiritigenin 20 mg/kg ip over time period indicated. *, significantly different from control group; **, significantly different from all other groups.

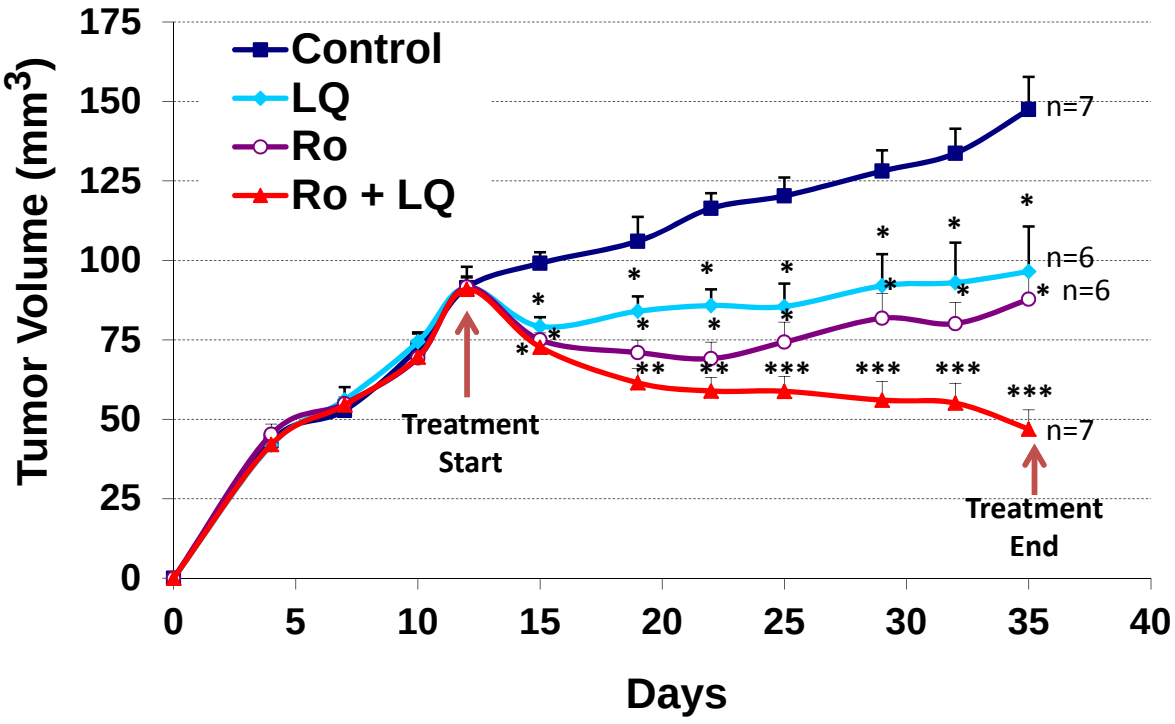


Fig 9B: Animal weight during drug treatment shown in Fig 9A.

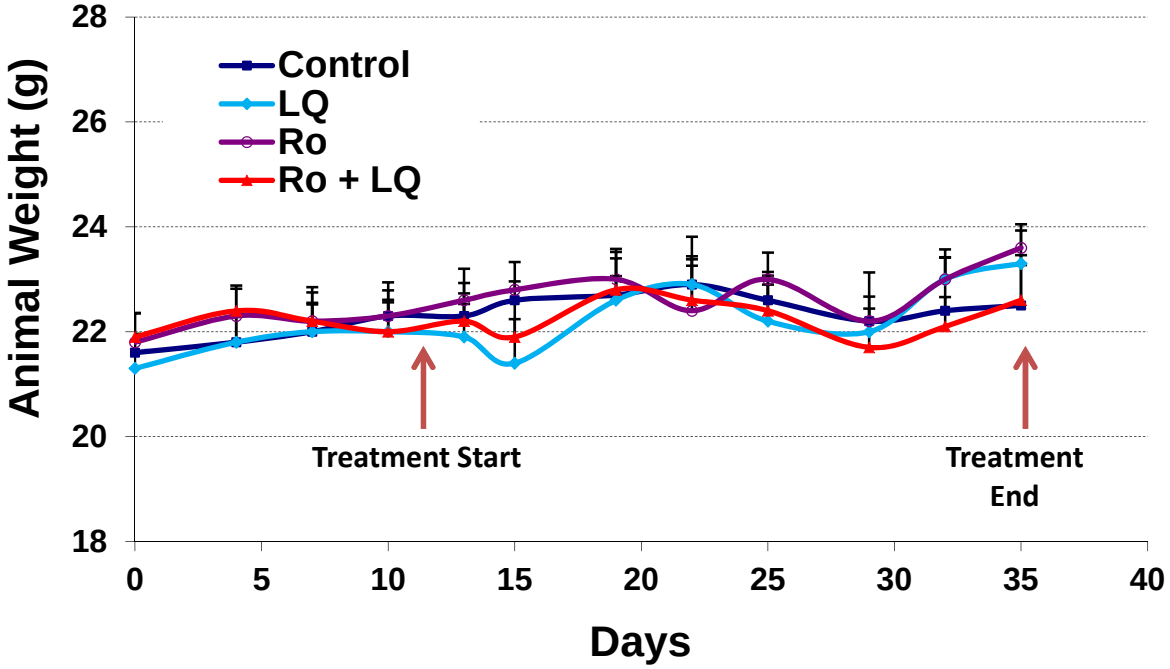


Fig 9C: Effect of RO 48-8071 combined with Liquiritigenin on clearance of BT-474 tumors in vivo.

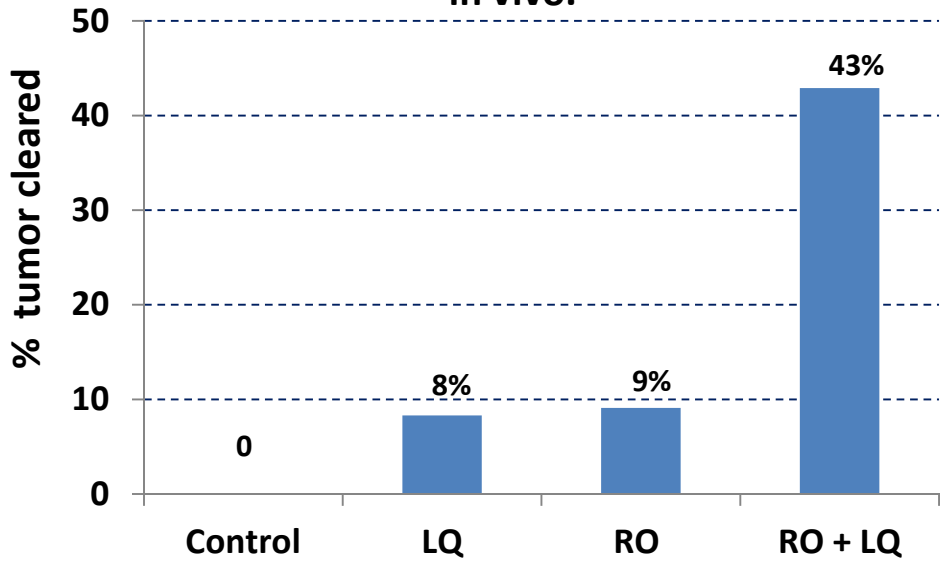


Fig 10A: Effect of Ro 48-8071 combined with Liquiritigenin on in vivo growth of MDA-MB-231 xenografts. Treatment was with RO 10 mg/kg iv + Liquiritigenin 20 mg/kg ip over time period indicated. *, significantly different from control; ** significantly different from all other groups (ANOVA)

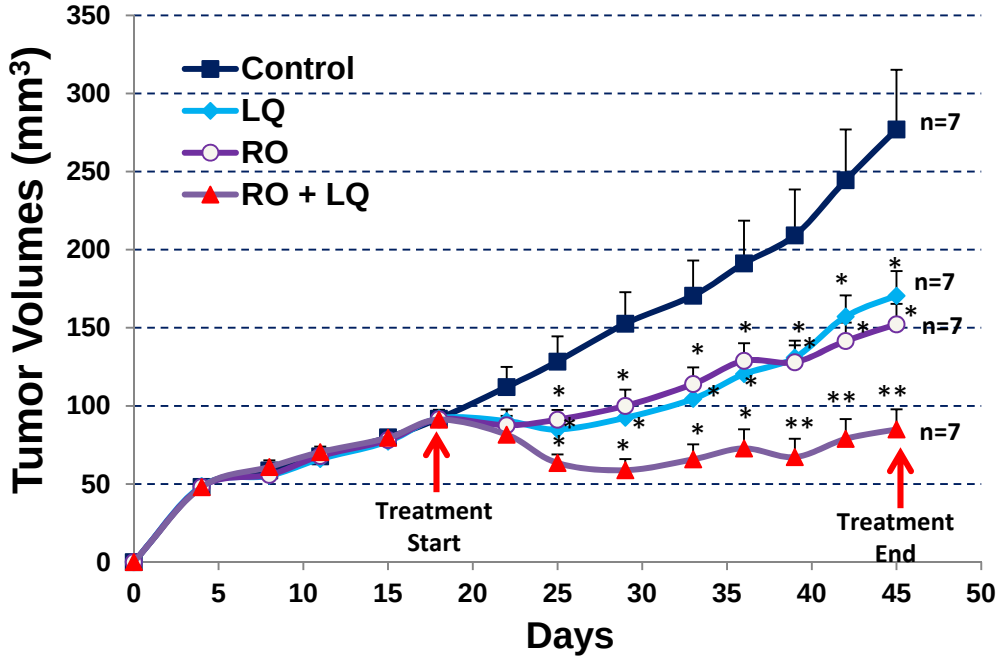
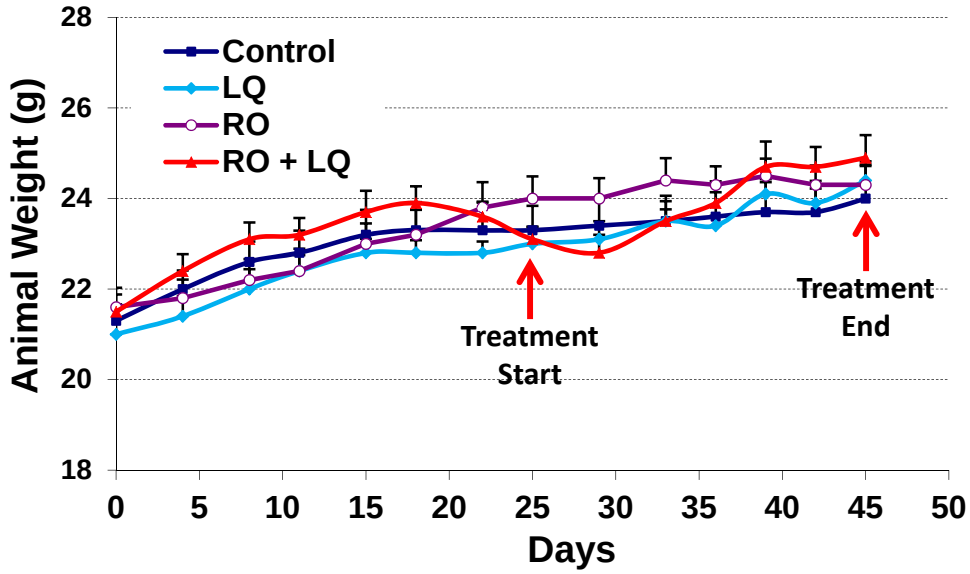


Fig 10B: Animal weight during drug treatment shown in Fig 10A



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PRECLINICAL STUDY

Cholesterol biosynthesis inhibitors as potent novel anti-cancer agents: suppression of hormone-dependent breast cancer by the oxidosqualene cyclase inhibitor RO 48-8071

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Abstract In most human breast cancers, tumor cell proliferation is estrogen dependent. Although hormone-responsive tumors initially respond to anti-estrogen therapies, most of them eventually develop resistance. Our goal was to identify alternative targets that might be regulated to control breast cancer progression. Sulforhodamine B assay was used to measure the viability of cultured human breast cancer cell lines exposed to various inhibitors. Protein expression in whole-cell extracts was determined by Western blotting. BT-474 tumor xenografts in nude mice were used for in vivo studies of tumor progression. RO 48-8071 ([4′-[6-(Allylmethylamino)hexyloxy]-4-bromo-2′-fluorobenzophenone fumarate]; RO), a small-molecule inhibitor of oxidosqualene cyclase (OSC, a key enzyme in cholesterol biosynthesis), potently reduced breast cancer cell viability. In vitro exposure of estrogen receptor (ER)-positive human breast cancer cells to pharmacological levels of RO or a dose close to the IC_{50} for OSC (nM) reduced cell viability. Administration of RO to mice with BT-474 tumor xenografts prevented tumor growth, with no apparent toxicity. RO degraded ERα while concomitantly inducing the anti-proliferative protein ERβ. Two other cholesterol-lowering drugs, Fluvastatin and Simvastatin, were less effective in reducing breast cancer cell viability and were found not to induce ERβ. ERβ inhibition or knockdown prevented RO-dependent loss of cell viability. Importantly, RO had no effect on the viability of normal human mammary cells. RO is a potent inhibitor of hormone-dependent human breast cancer cell proliferation. The anti-tumor properties of RO appear to be in part due to an off-target effect that increases the ratio of ERβ/ERα in breast cancer cells.

Keywords Breast cancer · Tumor progression · Cholesterol biosynthesis inhibitors · Estrogen receptor

Abbreviations

E	Estrogen
ER	Estrogen receptor
PR	Progesterone receptor
OSC	Oxidosqualene cyclase
RO	RO 48-8071 ([4′-[6-(Allylmethylamino) hexyloxy]-4-bromo-2′-fluorobenzophenone fumarate])
FBS	Fetal bovine serum
SRB	Sulforhodamine B
PI	Propidium iodide

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sc	Subcutaneous
iv	Intravenous
PBS	Phosphate-buffered saline
TBS-T	Tris-buffered saline containing 0.1 % Tween 20
ANOVA	Analysis of variance
SE	Standard error
DPN	2,3-bis(4-Hydroxyphenyl)-propionitrile
PHTPP	4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol
FACS	Fluorescence-activated cell sorting

Introduction

Estrogens (E) are essential steroid hormones that regulate sexual development and reproductive functions in humans. The diverse biological effects of E are mediated by the specific estrogen receptors (ER) ERα and ERβ [1–3]. Almost 70 % of human breast tumors express both ER and progesterone receptor (PR) and proliferate in response to the respective hormone [4–6]. At the cellular level, E and progestins stimulate cell proliferation and metastasis [4–7], promote angiogenesis [8], inhibit cell death [9, 10], and increase the risk of breast cancer in post-menopausal women on hormone replacement therapy [11–14]. ERα-positive breast cancers are usually treated with anti-estrogens and aromatase inhibitors, but resistance to these agents invariably develops during the course of therapy; these drug-resistant tumors then proliferate more aggressively than the drug-sensitive tumors from which they arose [15, 16]. Therefore, novel and more effective treatment strategies that could target ERs in hormone-dependent breast cancer are urgently needed.

Enzymes in the cholesterol biosynthetic pathway are attractive therapeutic targets for hormone-dependent breast cancer, because cholesterol serves as the metabolic precursor of endogenous steroid hormones, including those found in tumors [17, 18]. In addition, breast cancer cells have the capacity to synthesize cholesterol, and it is possible that endogenously produced cholesterol could contribute to the development of anti-hormone resistance [18, 19]. Statins, which are the most commonly used class of cholesterol-lowering drug, inhibit HMG-CoA reductase, an enzyme in the cholesterol biosynthetic pathway; however, certain undesirable side effects limit their long-term use for cancer therapy [20]. 2,3-Oxidosqualene cyclase (OSC) is an enzyme that acts downstream of HMG-CoA reductase to convert 2,3-monoepoxysqualene to lanosterol (a key step in the biosynthesis of cholesterol) [21–23]. While testing small-molecule inhibitors of OSC, we identified RO 48-8071 ([4′-[6-(Allylmethylamino)hexyloxy]-4-bromo-2′-fluorobenzophenone fumarate] (RO) [21–23] as a potent suppressor of breast tumor cell viability [24]. In the present study, we describe the anti-tumor effects of RO on ERα-positive tumors, both in vitro and in vivo. We observed that in addition to its recognized properties, RO also had off-target effects, degrading ERα while concomitantly inducing ERβ, the latter of which has been shown to block proliferation of breast cancer cells [25–28] and suppress tumor angiogenesis [29]. Consistent with these findings, we found that the anti-proliferative effects of RO were blocked by an ERβ-specific antagonist and ERβ-targeted siRNA. RO also induced apoptosis of breast cancer cells. Thus, RO exhibits unique anti-tumor properties, making it an exciting candidate compound for clinical management of breast cancer progression when used as mono-therapy and potentially in combination with ERβ-specific ligands.

Materials and methods

Cell lines and culture

ERα-positive breast cancer cell lines and normal mammary cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in phenol red-free DMEM:F12 medium (Invitrogen Corporation & Life Technologies, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA).

Reagents

RO 48-8071, Fluvastatin, Simvastatin, ICI 182,780, and U1866A were purchased from Sigma-Aldrich; RO analogs were provided by Roche Pharmaceuticals (Basel, Switzerland) and were synthesized as previously described [22, 30]. MG-132 was from Calbiochem; 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) and 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) were from Tocris Biosciences. Sixty-day release pellets containing 17-β-estradiol (1.7 mg) or placebo were obtained from Innovative Research of America (Sarasota, FL, USA). Antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), as were human ER-β-siRNA (sc-35325) and scrambled siRNA (sc-37007). Lipofectamine™, RNAiMAX, and Opti-MEM medium were obtained from Invitrogen Corporation & Life Technologies. RNAZol for RNA isolation was purchased from Molecular Research (Cincinnati, OH, USA).

Cell viability assay

The sulforhodamine B (SRB) assay was used to measure cell viability, as previously described by us [31].

Cell apoptosis and death assay

Cells were analyzed for apoptosis using the Annexin V-FITC Apoptosis Detection Kit (Biovision Research Products, Mountain View, CA, USA) as previously described [32].

In vivo breast tumor growth inhibition assays

All animal experiments were approved by the Institutional Review Committee. Female athymic nude mice (nu/nu, Foxn1), 5 to 6 weeks old and weighing 20–22 g, were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN, USA). Mice were implanted subcutaneously (sc) with pellets containing either 17-β-estradiol (1.7 mg/pellet, 60-day release) or placebo prior to inoculation of BT-474 breast cancer cells as previously described by us [33]. Tumor volumes were measured as described previously [33], and drug treatment was started when tumor volumes reached approximately 100 mm^3. Mice treated with RO received 5 or 10 mg/kg by intravenous (iv) injection of a 0.1 ml solution into the tail vein daily for 5 days, followed by an injection every other day for five additional treatments and then a final injection 2 h prior to sacrifice. Control mice received the same volume of phosphate-buffered saline (PBS) on the same schedule. Animals were weighed twice weekly throughout the study.

Tumors were collected following the last injection and processed for immunohistochemical analysis of ERα and ERβ as described previously [33, 34]. Quantitation of immunolabeled signal was achieved using a morphometric analysis program (FoveaPro 3.0, Reindeer graphics), on images photographed at 20× magnification as described earlier [34]. 3–4 Tumors/groups were analyzed for ER signal, and 2–3 representative sections were collected from each tumor. Results are expressed as area in square pixels.

Western blots

Whole-cell extracts were prepared with a nuclear extraction TransAm kit (Active Motif, Carlsbad, CA, USA) as described previously, and Western blotting was carried out as previously described [30, 32, 33].

siRNA knockdown

ERβ siRNA transfection was conducted following the manufacturers protocol (Santa Cruz). The transfection medium used was Opti-MEM, and transfection reagent was Lipfectamine RNAiMAX. The day before transfection, cells were seeded in 6-well plates at a density of 8×10^4 cells/well with 10 % FBS DMEM:F12 medium. Cells were incubated for 24 h with siRNA, after which 1 ml fresh 10 % FBS DMEM:F12 medium was added to each well. Cells were then incubated for another 24–48 h prior to treatment with RO.

Statistical analysis

Differences between groups or among groups were tested, respectively, using one-way analysis of variance (ANOVA) with repeated measures over time. The assumption of the ANOVA was examined, and a nonparametric measure based on ranks was used if needed. Values are reported as mean ± SEM. When ANOVA indicated a significant effect (F-ratio, $P < 0.05$), the Student–Newman–Keuls multi-range test was used to compare the means of the individual groups. Statistical analyses were conducted using SigmaStat software, version 3.5. For immunohistochemical analysis, data were analyzed using Kruskal–Wallis ANOVA, followed by Tukey's procedure as a post hoc test. For all comparisons, $P < 0.05$ was regarded as statistically significant. Values are reported as mean ± SEM.

Results

OSC inhibitors reduce cell viability of ERα-positive breast cancer cells but not normal mammary cells

Using several ERα-positive breast cancer cells, we tested the ability of four OSC inhibitors to reduce cell viability (Fig. 1a). While all four compounds reduced cell viability, RO 48-8071 and RO 61-3479 most effectively reduced the viability of BT-474, T47-D, and MCF-7 cells in a time- and dose-dependent manner. We selected RO 48-8071 (referred to as RO from this point forward) as the lead compound for further studies. RO also effectively reduced cell viability of HCC-1428 and ZR-75 cells (Online Resource 1). The IC_{50} values for the cell lines tested ranged from approximately 6–15 μM in a 24–48 h SRB assay (Table 1). Because the affinity of RO for OSC is in the nM range [22, 23], we examined whether a range of low doses of RO would affect cell viability over an extended period of time (7-day assay) similar to the effects observed for higher doses over a 24-h period. We found that RO concentrations as low as 1 nM effectively reduced BT-474 and MCF-7 cell viability in 7-day assays (Fig. 1b). To determine whether RO specifically reduces cancer cell viability, leaving normal cells unaffected, we conducted studies using normal AG11132A

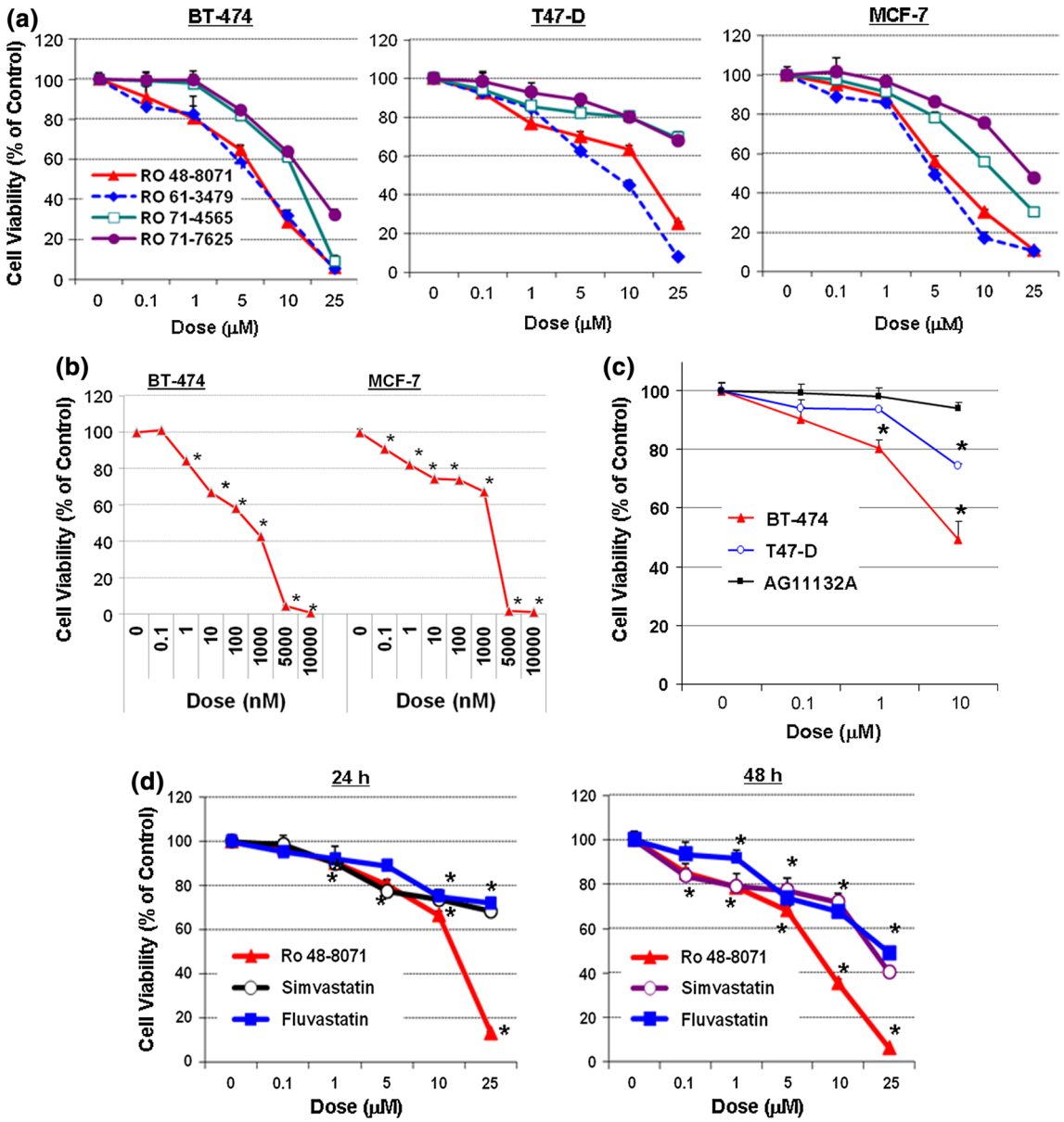

Fig. 1 OSC inhibitors reduce the viability of breast cancer cells but not normal mammary cells. **a** Breast cancer cells were incubated with pharmacological doses of indicated compounds for 48 h. **b** Breast cancer cells were incubated with low-dose (nM range) RO for 7 days. **c** Normal mammary cells (AG11132A) were treated with pharmacological doses of RO for 24 h and compared directly with the two cancer cell lines shown. **d** BT-474 breast cancer cells were treated with RO or the statins Simvastatin or Fluvastatin for 24 or 48 h. Cell viability was determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly different from control (set at 100 %) ($P < 0.05$ using ANOVA)

Table 1 IC_{-n} values of RO 48-8071 on breast cancer cell lines

Cell lines	IC_{50} (μM) (24 h)	IC_{50} (μM) (48 h)
BT-474	9.51 ± 0.05	6.06 ± 0.23
T47-D	11.53 ± 0.36	7.76 ± 0.29
MCF-7	12.32 ± 0.59	6.34 ± 0.34
HCC-1428	14.64 ± 0.42	11.58 ± 0.34
ZR-75	11.04 ± 0.29	7.63 ± 0.30

mammary cells. Concentrations of RO up to 10 μM reduced cancer cell viability, but had no effect on normal cells (Fig. 1c).

We also compared the ability of RO to reduce breast cancer cell viability with that of two other inhibitors of cholesterol biosynthesis (statins). The HMG-CoA reductase inhibitors Simvastatin and Fluvastatin also reduced cell viability; however, RO was more effective than either statins in 24- or 48-h assays (Fig. 1d).

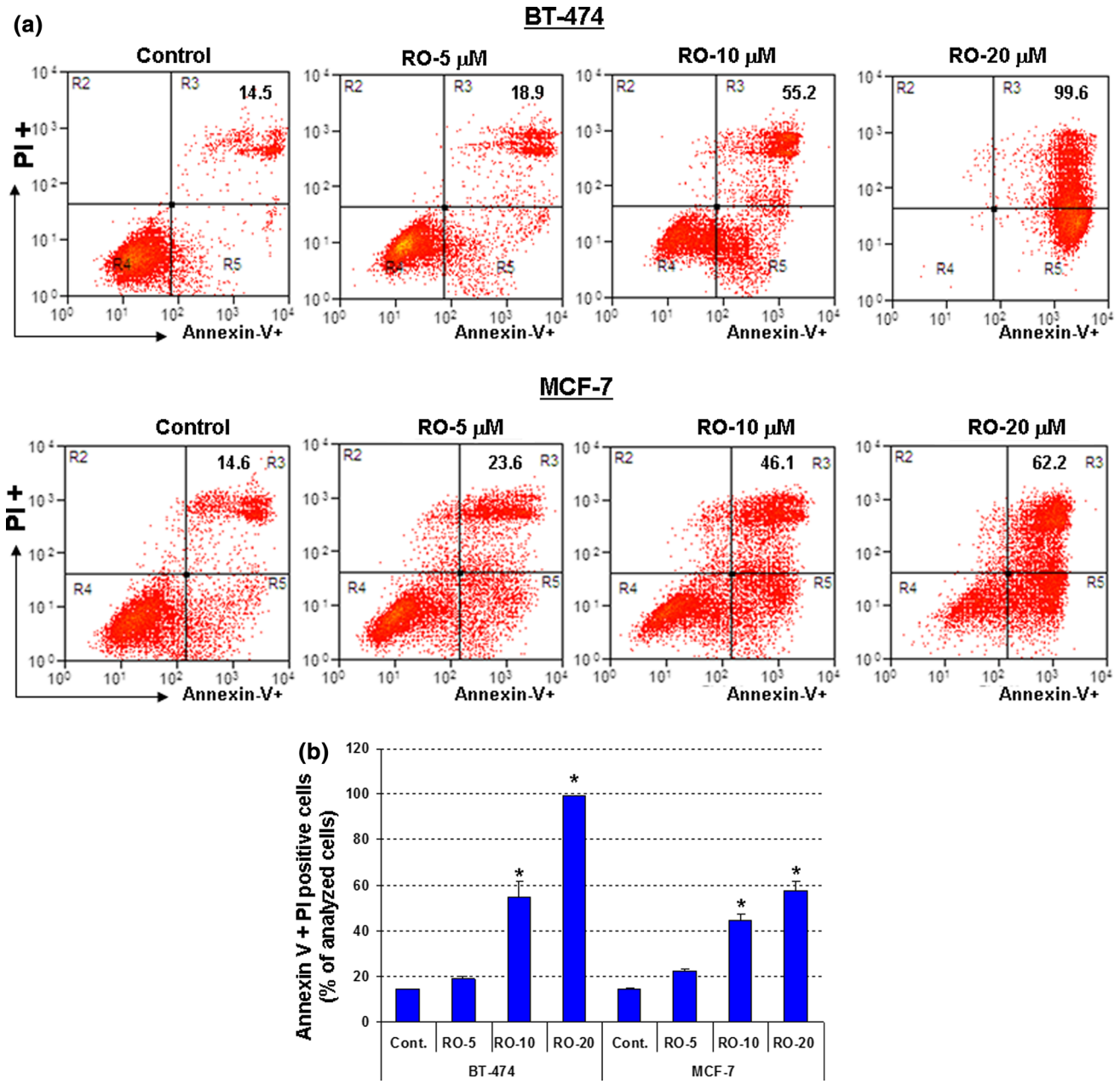

Fig. 2 RO induces apoptosis and cell death in breast cancer cells. **a** BT-474 and MCF-7 cells were seeded in 6-well plates overnight in 10 % FBS DMEM:F12 (1.5×10^5/well). After washing and replacement of media, cells were treated with 5, 10, or 20 μM RO or vehicle alone (control) for 24 h. Following treatment, cells were harvested and stained with annexin V-FITC and propidium iodide (PI). Fluorescence-activated cell sorting (FACS) analysis of 10,000 cells/sample was conducted. Quadrant R5 (*bottom right*) shows annexin V-positive (apoptotic) cells, and quadrant R3 (*top right*) shows annexin V-positive/PI-positive (dead) cells. **b** Quantitative data from FACS analysis. Values represent mean ± SEM ($n = 3$). *Significantly different from control ($P < 0.05$ using ANOVA)

RO induces apoptosis and cell death in breast cancer cells

In order to determine the mechanism by which RO reduced breast cancer cell viability, we treated BT-474 and MCF-7 cells for 24 h with 5, 10, or 20 μM RO. Cells were then collected, and the levels of apoptosis and cell death were determined. RO significantly induced apoptosis and cell death in both cell lines in a dose-dependent manner (Fig. 2a, b).

RO suppresses E-dependent proliferation of breast cancer cells in vitro and in vivo

Because E promotes proliferation of ERα-positive cells [1, 4], we examined whether RO reduces hormone-dependent

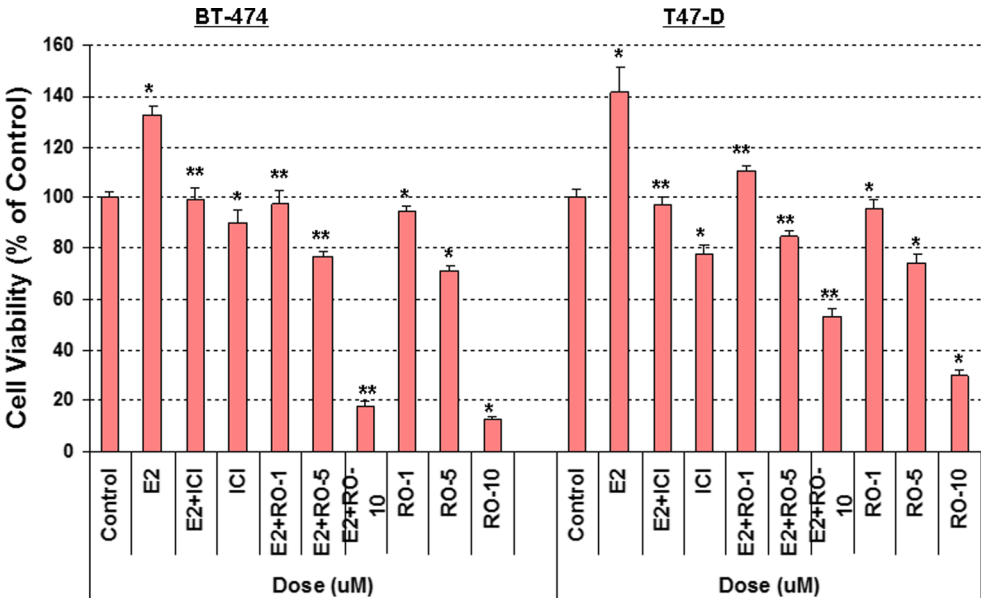

Fig. 3 RO suppresses Estradiol (E2)-induced proliferation of breast cancer cells. Breast cancer cells were treated with or without 10 nM E2 ± 1, 5, or 10 μM RO or 1 μM ICI 182,780 (ICI) for 24 h in 5 % charcoal stripped serum, after which cell viability was determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly different from control (set at 100 %); **significantly different from E2 ($P < 0.05$ using ANOVA)

proliferation of breast cancer cells. Using the anti-estrogen ICI 182,780 (which suppresses E-dependent cell proliferation [35, 36]) as our control ligand, we found that RO blocked E-dependent proliferation in four different breast cancer cell lines (Fig. 3 and data not shown for ZR-75 and MCF-7 cells). Furthermore, concentrations of RO that reduced E-induced cell proliferation also reduced cell viability in the absence of E.

Having demonstrated the effectiveness of RO in suppressing E-dependent breast cancer cell growth in vitro, we conducted studies to establish whether it had the same effect in vivo. We established estrogen-dependent BT-474 tumor xenografts in nude mice and began treatment with RO when the tumor volumes were approximately 100 mm^3. Compared with controls, the tumor burden of animals administered RO was significantly reduced (Fig. 4a). Furthermore, animal weights were unaffected by RO treatment, indicating that the compound was non-toxic at the dose administered (Fig. 4b). No changes in blood chemistry or evidence of cataracts was observed, as determined by CBW, Head Pathologist IDDEX RADIL (data not shown).

In order to determine the effects of RO on levels of ERα and ERβ protein expression in xenografts, we conducted immunohistochemical analysis of sections obtained from tumors collected at the end point in Fig. 4a. RO treatment resulted in significantly reduced levels of ERα within tumor tissue; however, ERβ was more resilient to depletion (Fig. 4c). While there was a trend toward elevated levels of ERβ in animals receiving 5 and 10 mg/kg, significance was attained in only the 10 mg/kg treatment group.

RO reduces levels of ERα protein and increases levels of ERβ protein in breast cancer cells in vitro

Because RO prevented E-induced cell proliferation and caused a loss of ERα protein, we determined whether it affected levels of ERα and ERβ in breast cancer cells in vitro. Pharmacological levels (25 μM) of RO reduced ERα in three breast cancer cell lines in a time-dependent manner. RO was most effective against BT-474 cells; most of the receptor was lost after just 3 h of exposure in these cells, compared with 6 h for other cell lines (Fig. 5a, upper panel). The loss of ERα following treatment with RO for 3 h (BT-474) or 6 h (T47-D and MCF-7) was dose-dependent (1–25 μM) (Fig. 5a, lower panel). Using BT-474 cells, we tested whether loss of ERα was due to proteasomal degradation. We found that this RO-mediated effect was dependent on ubiquitination, because treatment with MG-132, an inhibitor of proteasomal degradation, prevented receptor loss (Fig. 5b).

Importantly, when we examined ERβ levels in RO-treated breast cancer cells, we found that in a short-term assay, ERβ was increased in both BT-474 and T47-D cells in a time- and dose-dependent manner (Fig. 5c). RO also decreased expression of the survival protein Bcl-2 in breast cancer cells (Fig. 5d). Comparable results for changes in expression of ERα and ERβ in response to RO treatment were obtained in a longer term assay (up to 48 h), using lower concentrations of RO (0.1–10 μM) (Fig. 5e). Thus, our results indicate that treatment of breast cancer cells with RO leads to loss of ERα while simultaneously increasing ERβ. Using BT-474 cells, we found that even lower (nM) doses of RO used for an extended period of time (7 days) degraded ERα and induced

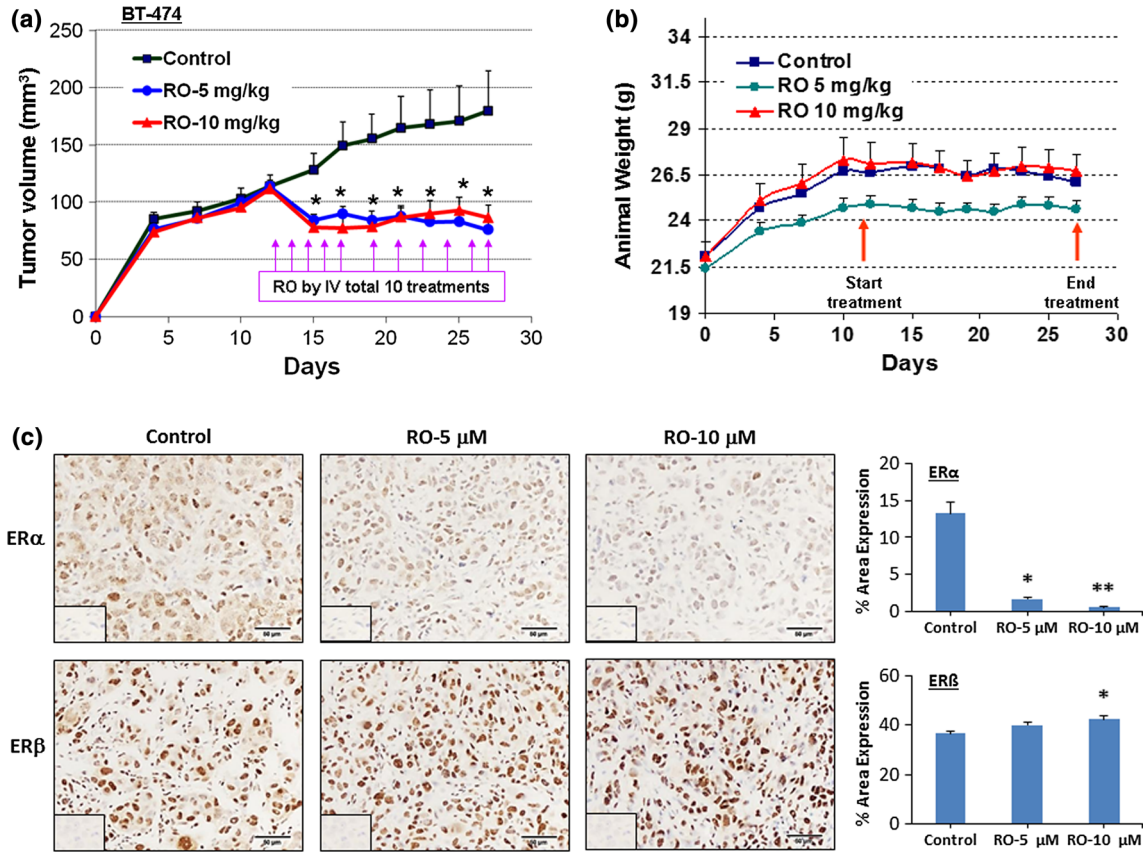

Fig. 4 RO suppresses growth of E-dependent xenografts in nude mice. **a** Six-week-old nude mice received an estradiol slow-release (1.7 mg/60-days release) or placebo pellet by sc implantation 48 h prior to injection with 5×10^6 BT-474 breast cancer cells in Matrigel:DMEM/F12 (4:1; [v/v]) on both flanks. When tumor volumes reached approximately 100 mm^3, animals were treated with RO (5 or 10 mg/kg) or the same volume of PBS (control) daily for 5 days, then every other day for five additional treatments by iv tail-vein injection; mice were given a final RO treatment 2 h before they were sacrificed. Values represent mean ± SEM ($n = 5$). *Significantly different from control ($P < 0.05$ using ANOVA). **b** Animal weight was monitored throughout the experiment. *Arrows* indicate duration of RO treatment. **c** Tumors were collected at end point as shown in **a** and processed for immunohistochemistry and data analysis as described in "Materials and Methods". *Insets* represent negative controls and *bars* represent 50 µm. RO reduced ERα and increased ERβ staining within tumors. *Indicates $P < 0.05$ compared with controls, **denotes significant difference compared with control and treatment with 5 µM RO

ERβ (Fig. 5f, upper panel). In addition to ERβ, p21, an apoptosis and cell-cycle arrest protein, was also induced under these conditions (Fig. 5f, lower panel). p21 was also reduced in T47-D cells (data not shown), suggesting that the effects of RO are not confined to one cell line. Thus, loss of ERα and ERβ induction by RO in breast cancer cells appears to be off-target effects that occur in response to both low and high doses of the OSC inhibitor. Exposure of BT-474 or T47-D breast cancer cells to 25 µM RO did not affect levels of mRNA for either receptor (data not shown), indicating that RO-induced changes in ER levels were independent of RNA transcription.

Finally, we examined the effect of RO analogs, a different class of OSC inhibitor (U18666A), and HMG-CoA reductase inhibitors (statins) on ERα and ERβ levels. When BT-474 cells were exposed to 25 µM RO, three analogs of RO, or U18666A, only the two RO analogs that were found to be most effective in reducing breast cancer cell viability (RO 48-8071 and RO 61-3479; Fig. 1a), caused a loss of ERα and increased ERβ (Fig. 5g). Of the two HMG-CoA reductase inhibitors tested (Simvastatin and Fluvastatin), only Fluvastatin decreased ERα levels. Neither statins elevated ERβ levels (Fig. 5h).

Modulation of ERβ activity modifies the anti-proliferative effects of RO on breast cancer cells

ERβ is known to play an anti-proliferative role in breast cancer cells [25–28, 37]. To determine whether induction of ERβ protein potentiates the anti-proliferative effects of RO, we treated BT-474 cells with RO in the presence

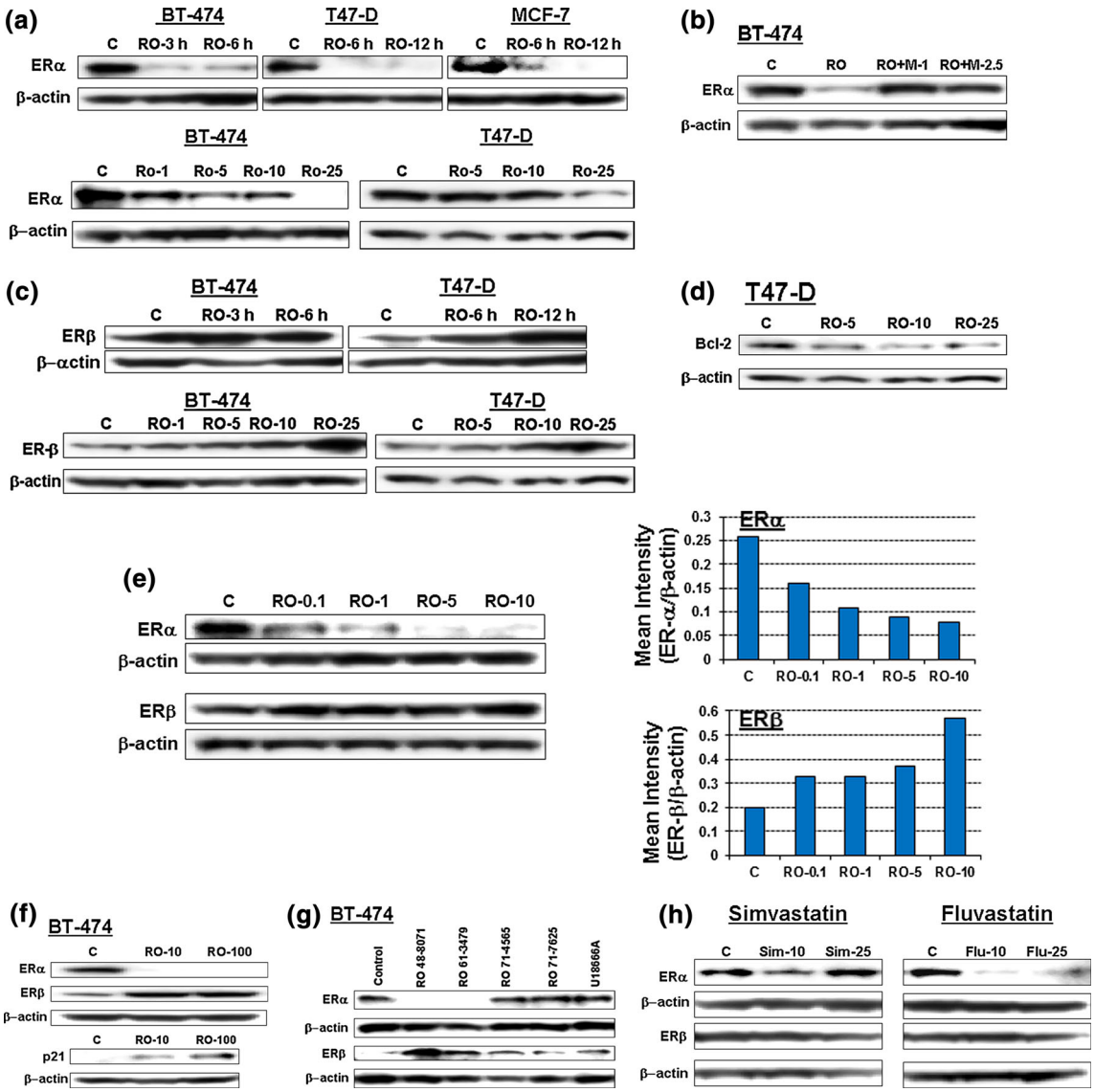

Fig. 5 RO decreases ERα and increases ERβ in breast cancer cells. **a** Breast cancer cells were treated with 0 (control; C), 1, 5, 10, or 25 μM RO for 3 or 6 h (BT-474) or 6 or 12 h (T47-D and MCF-7) in 5 % FBS DMEM:F12. *Upper panel*, all treatments with 25 μM RO; *lower panel*, BT-474 cells were treated for 3 h, and T47-D cells were treated for 6 h. **b** BT-474 cells were treated with 25 μM RO alone or in combination with 1 or 2.5 μM MG-132 (M) in 5 % FBS DMEM:F12 for 3 h. **c** Breast cancer cells were treated with 0 (control; C), 1, 5, 10, or 25 μM RO for 3 or 6 h (BT-474) or 6 or 12 h (T47-D and MCF-7) in 5 % FBS DMEM:F12. *Upper panel*, all treatments with 25 μM RO; *lower panel*, BT-474 cells were treated for 3 h, and T47-D cells were treated for 6 h. **d** T47-D cells were treated with 0 (control; C), 5, 10, or 25 μM RO for 6 h in 5 % FBS DMEM:F12. **e** BT-474 cells were exposed to sub-pharmacological levels of RO (0.1–10 μM) for 48 h. **f** BT-474 cells were treated with 10 or 100 nM RO for 7 days with a media change every 48 h containing fresh RO. **g** BT-474 cells were treated with the indicated compounds at 25 μM for 3 h. **h** BT-474 cells were treated with 10 or 25 μM Simvastatin or Fluvastatin (or vehicle, C) for 3 h. For all panels, whole-cell extracts were subjected to Western blotting to analyze protein expression, and levels of β-actin were assessed as a protein loading control. All experiments were conducted at least twice

of an ERβ agonist DPN. DPN enhanced the effects of RO on reducing the viability of breast cancer cells (Fig. 6a), suggesting that activation of ERβ is partially responsible for RO-mediated effects on breast cancer cell viability. Incubation of BT-474 cells with the ERβ antagonist PHTPP blocked RO-mediated reduction of cell viability (Fig. 6b), providing further evidence that ERβ plays a role in mediating the effects of RO on breast cancer cells. Interestingly, exposure of cells to PHTPP alone also increased cell viability. Similar observations were made with T47-D cells (data not shown).

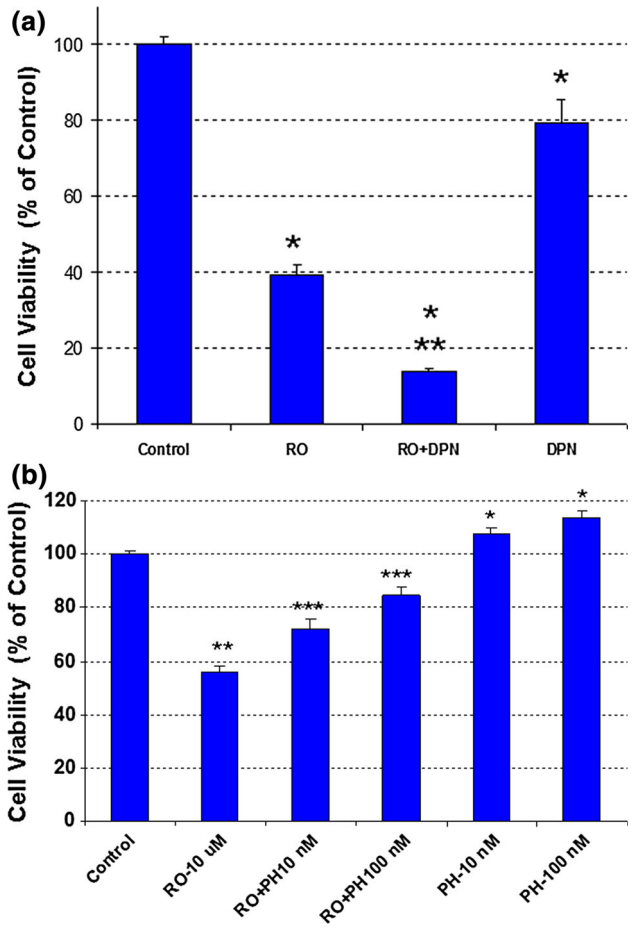

Fig. 6 Modulation of ERβ activity influences RO-mediated effects on breast cancer cell viability. **a** BT-474 cells were treated with 10 μM RO ± 1 μM ERβ agonist DPN or with 1 μM DPN alone (dose taken from ref. [50]) for 48 h. Cell viability was determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly different from control; **significantly different from RO-treatment and DPN-treatment groups ($P < 0.001$, ANOVA). **b** BT-474 cells were treated with 10 μM RO ± 10 nM or 100 nM ERβ antagonist PHTPP (PH) for 24 h. Cell viability was determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly increased compared with control (set at 100 %); **significantly decreased relative to control group; ***significantly different from RO-treatment group

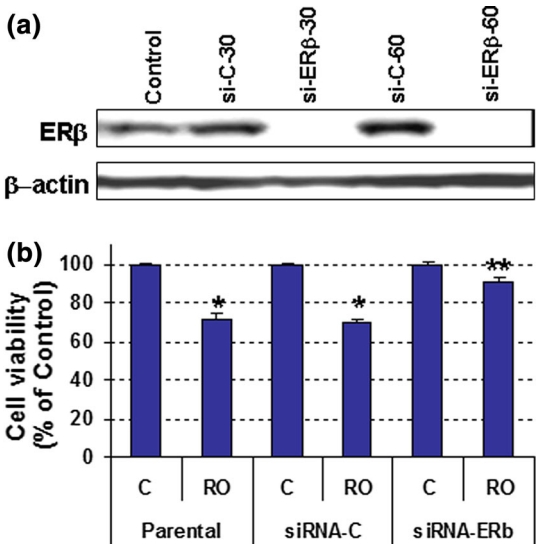

Fig. 7 ERβ knockdown blocks the anti-proliferative effects of RO in breast cancer cells. T47-D cells were transfected with 30 or 60 nM ERβ siRNA (si-ERβ) or scrambled siRNA (si-C) or transfection reagent alone (Control or C) for 72 h. **a** Whole-cell extracts were subjected to Western blotting to analyze ERβ expression. Levels of β-actin were assessed as a protein loading control. **b** Cells transfected with 60 nM siRNA (or T47-D cells transfected with transfection agent alone; parental cells) were treated with RO (10 μM) or vehicle alone (C) for 48 h and cell viability determined by SRB assay. Values represent mean ± SEM ($n = 6$). *Significantly different from vehicle control group; **significantly different from RO-treated samples in scrambled siRNA group and parental cell group ($P < 0.001$; one-way ANOVA)

Loss of ERβ reduces the anti-proliferative effects of RO in breast cancer cells

Finally, we used siRNA knockdown studies to determine whether RO-induced increases in levels of ERβ were responsible for anti-proliferative effects observed in breast cancer cells. ERβ siRNA but not the scrambled siRNA control effectively knocked down ERβ expression (Fig. 7a). Treatment with RO resulted in a loss of cell viability in both control samples and cells treated with scrambled siRNA. However, when cells were exposed to siRNA specific for ERβ, RO was unable to reduce cell viability to the same extent as it did in control and scrambled siRNA-treated cells (Fig. 7b). These findings support the idea that induction of ERβ is at least partially involved in mediating the effects of RO on reducing cell viability in breast cancer cells.

Discussion

Hormone-dependent breast cancer is the most common type of clinically observed mammary cancer [1, 4, 6]. Although a number of anti-hormonal treatment strategies are currently employed to control progression of the disease, drug-resistant tumors that continue to express ER frequently emerge [1, 4]. As a consequence, studies are ongoing whose goal is to identify new compounds with the ability to control ERα-dependent proliferation in breast tissue and thereby prevent tumor progression. While conducting studies in breast cancer cells to determine the anti-proliferative capacity of cholesterol biosynthesis inhibitors, we discovered that analogs of RO, a class of compound that blocks OSC activity, also down-regulated ERα. Furthermore, RO compounds simultaneously up-regulated the druggable anti-proliferative protein ERβ [25–28, 37], thus

negating the concerns that loss of ERα could lead to hormone-resistant tumors.

We examined the effects of four different RO analogs on ERα-positive breast cancer cell lines. All of them reduced the cell viability, with two particular compounds more potent than the others. We subsequently characterized the effects of RO 48-8071 (RO) as the lead compound on breast cancer cell proliferation and tumor development. RO effectively reduced tumor cell viability in short-term assays (IC_{50} values between 6 and 12 μM; SRB 48-h), while lower concentrations (nM) of RO significantly suppressed the viability of tumor cells in longer term (7 day) assays. We also observed that concentrations of RO up to 10 μM had no effect on the viability of normal mammary cells, suggesting that its in vitro effects are specific to breast cancer cells. Consequently, we propose that since RO appears to be non-toxic to normal cells, it might be used to target tumors with little risk of patient toxicity. Subsequent in vivo studies provide evidence which further supports the use of RO as a therapeutic agent with little or no risk of toxic side effects. Although it is unlikely that RO binds directly to ER due to strict structural requirements for ER-ligand interactions, we will determine whether it binds directly to ERα and ERβ in future studies using competition assays.

In order to compare the effects of RO with the more widely tested HMG-CoA reductase inhibitors (statins), we treated cells with Simvastatin and Fluvastatin, both of which effectively reduced breast cancer cell viability, though with less potency than RO. Our findings, therefore, suggest that RO is more effective than statins at inhibiting breast cancer cell proliferation.

RO suppressed E-induced proliferation of five breast cancer cell lines, including BT-474 cells, which are tamoxifen resistant and which express high levels of HER-2/neu. Tumors that are high in Her2/neu expression have poor prognosis [38, 39]. We administered RO to nude mice bearing BT-474 cell-derived xenografts grown in response to implanted E-containing pellets and observed suppression of tumor growth. This suggests that RO could be an effective means of suppressing cells that are resistant to anti-hormones, though this possibility remains to be tested. Studies are currently underway to determine whether administration of higher levels of RO might promote complete xenograft regression without toxicity.

Since RO suppressed the growth of E-dependent breast cancer cells, we conducted studies aimed at determining whether the OSC inhibitor affects ERα levels. We found that levels of ERα were indeed reduced dramatically in response to RO, in a time- and dose-dependent manner both in vitro and in vivo. BT-474 cells were most sensitive to RO, in accord with their sensitivity in SRB assays. Further in vitro studies showed that the proteasome inhibitor MG-132 completely blocked receptor loss, indicating that RO induces proteasome-mediated receptor degradation. Ubiquitination has previously been shown to control ER degradation [40]. ERα mRNA synthesis was not reduced by RO treatment, suggesting that the loss of ERα is a post-transcriptional event. The term selective ER down-regulator or degrader has been used to describe the effect of therapeutic agents that degrade ERα, and RO seems to be another member of this class [41, 42]. Previously, the anti-estrogen ICI-182,780 has been shown to cause a similar loss of ERα in breast cancer cells [41]; however, its use in long-term treatment is restricted due to bone-related toxicities in post-menopausal women [43]. Further studies are needed with RO to determine its effect on bone after a long-term use.

Importantly, we found that as well as degrading ERα, pharmacological concentrations of RO concomitantly increased ERβ levels in vitro. However, while induction of ERβ was significant in vitro, its up-regulation was not as robust in vivo. This was most likely due to tumors being collected at the end point, several days after the initial treatment of nude mice bearing BT-474 xenografts. It is, therefore, likely that we missed the higher levels of ERβ, which were subsequently lost when cells expressing elevated levels of ERβ underwent apoptosis. However, this requires confirmation by collection of tumors a few days after initial treatment with RO and assessment of ERβ induction. The consistent in vitro induction of ERβ in various cell lines was most likely due to short-term exposure to the drug. In any event, the loss of ERα over time leads to a high ratio of ERβ to ERα, a scenario which has been shown to inhibit tumor cell proliferation [44]. We further characterized the in vitro effect of RO by real-time PCR analysis and found that the OSC inhibitor did not affect levels of ERβ mRNA. Thus, it would appear that RO likely stabilizes ERβ protein over time. In future studies, we will examine in more detail just how RO influences ERβ protein stability. Because BT-474 cells were most sensitive to the anti-proliferative effects of RO, we exposed this cell line to lower levels of RO and assessed ERβ induction. Doses of RO as low as 100 nM induced ERβ, while simultaneously degrading ERα, while in a 7-day assay, 10 nM RO completely eliminated ERα, while also inducing ERβ (Fig. 5f).

Of the several RO analogs tested, the two that reduced breast cancer cell viability most effectively also potently reduced ERα levels while at the same time inducing ERβ. Those that did not degrade ERα still induced ERβ to some extent. Based on these findings, we conclude that increased ERβ is the predominant off-target factor that accounts for loss of breast cancer cell viability following exposure to analogs of RO at least in vitro. Interestingly, HMG-CoA reductase inhibitors demonstrated a variable response;

while Simvastatin did not degrade ERα to the same degree as the two most effective RO analogs, Fluvastatin exerted a comparable effect in this regard. Neither Simvastatin nor Fluvastatin induced ERβ. It is clear that the two classes of cholesterol biosynthesis inhibitor exert differential effects on the ratio of ERα and ERβ in breast cancer cells. This ratio has been shown to be an important predictor of cell growth; a high ratio of ERα/ERβ is proliferative, whereas increased expression of ERβ is associated with loss of tumor cell proliferation [45–48].

To further confirm that ERβ is at least partially responsible for loss of breast cancer cell viability, we blocked receptor activity using PHTPP, a selective ERβ antagonist. PHTPP suppressed the anti-proliferative activity of RO in a dose-dependent manner. We further confirmed the important role played by ERβ in reducing cell viability by exposing breast cancer cells to DPN, an ERβ-specific agonist. When administered individually, both RO and DPN inhibited breast cancer cell viability. However, when a combination of the two compounds was given, their inhibitory effect was additive, an outcome that may be due to increased cellular levels of ERβ in response to RO. Down-regulation of ERβ by siRNA significantly reduced the anti-proliferative effects of RO, providing further evidence of the importance of ERβ in mediating RO effects on breast cancer cell viability. It therefore appears likely that drugs that increase ERβ activity in breast cancer cells could be made even more effective when administered in conjunction with RO. The development of therapeutic regimens using a combination of two agents might make it possible to manage disease using lower levels of both, reducing the likelihood of toxic side effects that result from current therapeutic modalities [49].

In summary, the data presented in this manuscript strongly suggest that, in addition to its ability to suppress cholesterol biosynthesis, the OSC inhibitor RO exerts a powerful anti-tumor effect by the off-target loss of ERα and induction of the anti-proliferative protein ERβ. The loss of ERα but not ERβ in vivo leads to a large increase in the ERβ/ERα ratio, which could be responsible for tumor loss [43–48]. In addition, in vitro data show that ERβ can promote some of the anti-tumor properties of RO. Thus, we propose that ERβ is at least partially responsible for the observed suppression of breast cancer cell viability and suggest, therefore, that combination therapy using inhibitors of cholesterol biosynthesis (such as RO) together with commonly used chemotherapeutic drugs could prove beneficial as a means by which to suppress breast cancer progression. We are currently conducting studies to determine the effectiveness of such combination therapies.

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Conflict of interest JDA is an employee of F. Hoffmann-La Roche AG. All other authors declare that they have no conflict of interest.

Ethical standards The experiments comply with the current laws of the country in which they were performed (USA).

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Poster Presentation

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Oxidosqualene Cyclase Inhibitor Suppresses Transcriptional Activity of Estrogen Receptor-α in Human Breast Cancer Cells

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Cholesterol is the synthetic precursor of steroid hormones such as estrogens and progestins, which control the growth of many types of human breast cancer. Enzymes responsible for converting cholesterol into steroid hormones are present within breast tumor cells, resulting in local estrogen production. This could lead to tumors becoming resistant to anti-estrogen therapy. In this study we determined whether oxidosqualene cyclase (OSC), an enzyme in the cholesterol biosynthetic pathway, might be targeted to suppress progression of breast cancer cells. Using in silico analysis we previously identified the OSC inhibitor RO 48-8071 (RO), as a potential ligand which could be used to control the progression of estrogen receptor-alpha positive (ERα +ve) breast cancer cells. However, real-time PCR analysis of mRNA from human breast cancer biopsies obtained at various stages of disease did not identify significant differences in OSC expression between tumor tissues or between tumor and normal mammary cells. Nevertheless, since RO reduced tumor cell viability, we examined other potential targets by which it might exert its anti-proliferative effects. Since the growth of hormone-responsive tumors is ERα-dependent, we determined whether RO affected ERα. Using non-human mammalian cells engineered to express human ERα protein and an ERα–responsive luciferase promoter (Indigo Biosciences) we found that RO inhibited 17β-estradiol (E2)-induced ERα responsive luciferase activity. Inhibition was dose-dependent, with an IC_{50} of approximately 10 μM under conditions that were non-toxic to the cells. In order to determine whether RO influenced the biological activity of ERα, we selected treatment conditions (5 μM RO, 16-18h) that did not affect cell viability or influence ERα protein levels. We then treated BT-474 breast cancer cells with 10 nM E2 ± 5 μM RO for 16-18 h and used Western blotting to measure levels of PR, an estrogen responsive gene. RO reduced PR levels in BT-474 cells, confirming that it blocks ERα activity in tumor cells. Real-time PCR and Western blotting revealed no effect of RO on levels of either ERα mRNA or protein. Our findings demonstrate that an important means by which RO suppresses hormone-dependent growth of breast cancer cells is through its ability to arrest the biological activity of ERα. We suggest therefore that our studies support further investigation of RO as a potential therapeutic agent for use against hormone-dependent breast cancers.

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